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DEFENSE TRADEOFFS IN FLESHY FRUITS: EFFECTS OF RESOURCE VARIATION ON GROWTH, REPRODUCTION, AND FRUIT SECONDARY CHEMISTRY IN *Solanum carolinense*

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Abstract—A set of clones of 10 maternal plants was grown for three successive years (1998–2000) under two nitrogen treatments and two water treatments. Path analysis revealed strong direct and indirect effects of nitrogen treatment on growth and reproduction, but fruit morphological and chemical variables were not strongly affected. Fruit pulp chemistry varied only slightly across treatments despite the large differences in growth and reproduction associated with resource variation. Leaf and ripe fruit chemical contents were not significantly correlated across treatments, and maternal plants, and leaf chemical variables did not help explain fruit chemical variation when included as covariates in ANCOVA analyses, suggesting no physiological constraints of leaf chemistry on ripe fruit chemistry. Results suggest that, while maternal plants may vary somewhat in fruit chemistry, and fruit chemistry may vary somewhat depending upon environmental conditions, levels of primary and secondary metabolites within fruits are not best explained by supply-side hypotheses. Ripe fruit chemistry remained relatively constant in the face of drastically changing resource levels, suggesting an adaptive function and supporting the Defense Tradeoff hypothesis. Fruit quality, both in terms of nutritional make-up and putative defensive properties, was maintained despite strong effects on plant growth and reproduction. Because glycoalkaloids are general defense compounds, we conclude that ripe fruit chemistry most likely reflects a balance between selection for attraction of seed dispersers and defense against pests and pathogens.

Key Words—Defense tradeoffs, frugivory, growth and reproduction, nitrogen supplementation, optimal defense, physiological constraints, plant secondary metabolites, ripe fruit chemistry and morphology, *Solanum carolinense.*

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INTRODUCTION

It is well known that plants can respond physiologically to environmental factors, and that these changes can affect the actions of organisms with which they interact (Tuomi et al., 1984; Waterman and Mole, 1989). However, the degree to which the chemistry of ripe fleshy fruits exhibits such biochemical responses is practically unknown outside of horticultural species (e.g., tomatoes, wine grapes). Such responses have implications with respect to frugivory and seed dispersal, as well as for models attempting to explain the evolution of fleshy fruit traits. Using an herbaceous perennial plant, *Solanum carolinense* L., we address this issue by focusing on three questions: 1) How does variation in nitrogen (N) availability affect plant growth, reproductive output (fruit number and size), and fruit pulp primary (nutrient) and secondary (phenolic, glycoalkaloid) chemistry? 2) Does N supplementation affect nutritional (proteins, sugars) and putatively defensive (phenolics, glycoalkaloids) metabolites in a manner consistent with the adaptive Defense Tradeoff Hypothesis (an Optimal Defense hypothesis), or with nonadaptive, supply-side hypotheses? 3) Do strong correlations exist between levels of leaf and fruit chemicals, as suggested by the nonadaptive, supply-side Physiological Constraints hypothesis?

The Optimal Defense (OD) hypothesis (Rhoades, 1979; Berryman, 1988) is receiving renewed attention recently, due partly to the inconsistent results obtained from many studies focusing on various supply-side hypotheses, most notably the Carbon-Nutrient Balance hypothesis (CNB hypothesis; Bryant et al., 1983; Stamp, 1992; Hamilton et al., 2001). The OD hypothesis suggests that, because defenses are presumably costly, plants should allocate them in proportion to the potential fitness value of a particular plant part, the cost of the defense, and the defensive advantages gained by such allocation. Supply-side hypotheses, on the other hand, are examples of nonadaptive (physiological or functional) models. Such models assume that the supply of substrates (e.g., nutrient availability) is the primary controlling factor influencing quantitative variation in secondary metabolites. The CNB hypothesis, for example, predicts that plants will allocate relatively more carbon resources to N-containing secondary metabolites when the plant has an abundance of N at its disposal (Muzika, 1993; Gebauer et al., 1998; Mutikainen et al., 2000). Conversely, the OD hypothesis is considered an adaptive, demand-side hypothesis, which assumes that demands placed upon tissues for defense, and the selective value of that tissue are the primary factors influencing quantitative variation in secondary metabolites. While the OD hypothesis was originally framed from a strictly defensive perspective, it should also apply to metabolites that function in the attraction of beneficial organisms (e.g., pollinators, seed dispersers).

The Defense Tradeoff (DT) Hypothesis (Cipollini and Levey, 1997b; Cipollini, 2000) is an example of an OD-type hypothesis formulated specifically

to predict the allocation of secondary metabolites in the pulp of fleshy, vertebrate-dispersed fruits. This hypothesis suggests that quantitative variation in ripe fruit chemistry can be explained as a balance between selective pressures favoring attraction of dispersers, and the attraction and deterrence of pests and pathogens. The allocation of secondary metabolites in ripe fruits, thus, represents a compromise between defensive and attractive functions, and selective pressures of various seed-dispersing and non-seed-dispersing consumers serve to mold and maintain the chemical make-up of the fruit pulp. Support for the DT hypothesis in ripe fruits may be inferred if plants maintain relatively consistent ripe fruit quality (size, seed number, in addition to pulp chemistry) in the face of drastic changes in resource levels, and weak correlation (if any) between leaf and ripe fruit chemistry. If this is seen, it may be assumed that the particular chemical profile of the ripe fruit has a selective advantage—it is unlikely to be an accident of history or physiology.

The Physiological Constraints (PC) hypothesis is a nonadaptive, supply-side hypothesis, also formulated to predict the allocation of secondary metabolites in ripe fleshy fruits (Cipollini and Levey, 1998; Eriksson and Ehrlen, 1998). This hypothesis suggests that both historical effects of phylogeny and selection for leaf defense have resulted in variation among plant species in leaf and unripe fruit toxicity (leaves being no different physiologically or ecologically from unripe fruits). Ripe fruit toxicity follows as a consequence of physiological constraints affecting the removal of toxins from fruits as they ripen (Eriksson and Ehrlen, 1998). As such, plants or plant species with high levels of toxins in the leaves are predicted to have high levels of those same toxins in the ripe fruits. Correlations among species in leaf and ripe fruit chemistry follow as a consequence of correlations within species (as species diversification occurs within plant lineages). Support for the PC hypothesis may be inferred if ripe fruit chemistry within a species is strongly affected by variation in plant nutrient availability, and if leaf and fruit chemical content is strongly correlated (both within and among species). If resource variation strongly affects fruit chemistry (e.g., if N-containing compounds rise in proportion to available N), plants would have little control over the attractive and defensive characteristics of the fruits. Similarly, if leaf chemistry strongly constrains the level of toxins within the ripening fruits, plants would have little control over factors influencing fruit removal by dispersers or defense against pests.

While looking for qualitative differences between leaf and ripe fruit chemistry is clearly one way of addressing the PC hypothesis (cf., Cipollini and Levey, 1998; Cipollini, 2000), an equally valid approach is to look for the predicted quantitative relationships. This is because many secondary metabolites *do* occur in both leaves and ripe fruits of the same species, albeit at variable levels. The selective value of retaining significant levels of such compounds in ripe fruits is the central question upon which we focus, rather than the question as to whether the presence of a particular metabolite in ripe fruits is an "adaptation" in the strict sense (Cipollini and Levey, 1998). Why do many ripe fruits contain high levels of certain types

of secondary metabolites? Is it a simple consequence of physiology, or are such quantitative patterns maintained via selective pressures? These questions do not apply exclusively to the production of ripe fruits high in putatively toxic substances, but apply to any and all compounds retained or produced following ripening. Furthermore, the retention of secondary compounds in ripe fruits is not uncommon, as had been previously assumed (Cipollini and Levey, 1997b; Cipollini, 2000).

The chemical characteristics upon which we focus include both potentially attractive and defensive substances. Assuming substrate-level control, we expected N-containing compounds (protein and glycoalkaloids) to increase in response to increased N availability. Protein may positively affect feeding rates and food choices of both pests and frugivores (e.g., Herbst, 1986; Hare, 1987; Pryor et al., 2001), whereas glycoalkaloids (N-containing steroidal bases) are active against a variety of organisms ranging from birds and mammals to insects and fungi (Hare, 1983; Tingey, 1984; van Gelder, 1990; Cipollini and Levey, 1997a,c). We also examined strictly C-based compounds (phenolics and sugars), but made no specific predictions about the effects of N supplementation. Phenolics, a group of miscellaneous aromatic compounds ultimately derived from cinnamic acid, may negatively influence feeding and relative growth rates of animals and pests through a variety of mechanisms (Swain, 1979; Ozawa et al., 1987; Cipollini and Stiles, 1991). Because of their caloric content and sweetness, sugars are expected to positively affect feeding rates and food choices of both frugivores and pests (Levey, 1987).

METHODS AND MATERIALS

Experimental Planting Design. In the fall and winter of 1997–98, 12 clones from each of 10 different maternal plants (Table 1) were propagated from root cuttings under greenhouse conditions. To maximize potential genetic variation

TABLE 1. SOURCES OF ORIGINAL *S. carolinense* MATERNAL PLANTS[a]

Code	Date	Collection location (USA)
AUG	1993	Augusta, Georgia
EAST	1991	Easton, Maryland
HOW	1991	Howard County, Maryland
KENT	1991	Kent Island, Maryland
N2O	1991	Smithsonian Environmental Research Center, Edgewater, Maryland
SACC	1991	Conservation and Research Center, Front Royal, Virginia
SEN	1991	Seneca Rocks, West Virginia
SERC	1991	Smithsonian Environmental Research Center, Edgewater, Maryland
TIFT	1993	Tifton County, Georgia
TWIN	1991	Kent Island, Maryland

[a] Collections of root-stock from a single plant were made at sites no closer than several km apart.

among the maternal plants, plants were collected from throughout the natural range of *S. carolinense*, from sites separated by several to hundreds of kilometers. In April 1998, each of the 120 clones was transplanted into a 12-l pot filled with a sandy-loam-clay soil mixture made to mimic a typical southeastern U.S. soil. The pots were arranged in a fully randomized array with pots 1 m apart, surrounded by a 2.5-m high wire mesh fence designed to deter mammalian herbivores, but to allow access by pollinating bumblebees. Three clones from each maternal plant were subjected to the following manipulations:

a. Control (C): Received ambient rainfall only; to minimize drought stress, watered only if any of the plants in the entire array appeared wilted (when this was done, all 120 plants were watered); b. Water Addition (W): Same as control group, but watered to complete soil saturation three additional times per week; c. Nitrogen Addition (N): Same as control group, but 10 g dry ammonium nitrate fertilizer added four times during each year (mid-April, -May, -June, and -July); d. Water and Nitrogen (N + W): Combination of water and N treatments. Pots were randomly rearranged on an annual basis, and treatments were continued on the same set of plants until the fall of 2000. The water addition treatment was used primarily because of the potential effect of water availability on nitrogen availability.

Quantification of Growth and Reproductive Variables. Plants were allowed to grow, set fruit, and senesce naturally each fall. At the end of each fruit-ripening season (late November 1998, 1999, 2000), all ripe fruits were collected, counted, and weighed, and all aboveground stems were collected, dried to constant mass, and weighed. In 1998, five of the 10 clones produced ripe fruit under all treatment conditions, and in 1999 all plants produced ripe fruit. Fruits collected from these plants were flash-frozen, and freeze-dried. Freeze-dried whole fruit samples were crushed and screened to remove seeds, and the pulp was pulverized with mortar and pestle into a 40-mesh powder. In August of 1999, 10 whole leaves were removed from each plant (less than 5% of total foliage), flash-frozen, freeze-dried, and ground to a 40-mesh powder using a Wiley Mill.

Fruit Chemical Parameters. We analyzed fruit pulp samples from 1999 for total dietary protein (PROT), total phenolics (PHEN) and total sugars (SUG). PROT was analyzed using the Bradford (1976) assay as modified for plant material by Jones et al. (1989) using ribulose 1,5 biphosphate carboxylase/oxygenase (the most prevalent protein in plant leaves) as an external standard. PHEN was analyzed using the Coomassie Brilliant Blue technique (Budini et al., 1980) with modifications of Graham (1992) and using chlorogenic acid (the most prevalent phenolic in horsenettle plants) as an external standard. SUG was analyzed by hot-ethanol extraction followed by anthrone analysis (Smith, 1981). Fruit pulp samples from 1998 and 1999 were analyzed for total glycoalkaloids (TGA) using the method of Birner (1969) with modifications of van Gelder (1984) using solasodine (the sole N-containing steroidal base in horsenettle plants) as an external standard. To address

the issue of physiological constraints on fruit metabolites, we also measured total PROT, PHEN, and TGA in freeze-dried leaf material collected in 1999.

Statistical Analyses. Data for duplicate chemical analyses differing by less than 5% were averaged to produce a single value for each chemical parameter for each sample. We included these variables and other plant growth and reproductive variables in the following statistical analyses (following log- or arc-sine transformation as necessary to achieve approximate normal distributions, all statistics were calculated using SPSS for Windows (SPSS, Inc., 1999).

We included plant growth and reproductive variables in a path analysis (Schemske and Horvitz, 1988) designed to determine both the direct and indirect effects of plant treatment on growth and reproduction. As plant treatment, growth, and reproduction in one year were expected to affect plant growth and reproduction in subsequent years, these variables acted as both independent and dependent variables in a path analysis model. Path analysis uses standardized regression coefficients summed along paths of a logical cause-and-effect model to additively derive path coefficients. Path analysis is particularly well suited for the analysis of complex models in which dependent variables may subsequently serve as independent variables, allowing the analysis of both direct and indirect effects on variables of interest. In our case, all variables that preceded other variables in occurrence by 1 year or less served as independent variables for later dependent variables. For example, aboveground biomass in 1998 was expected to affect aboveground biomass in 1999 (1 year later), but not in 2000 (2 years later). We first specified a full path analysis model (including all variables), and report simplified results showing only paths with regressions that were significant (Figure 1).

Fruit pulp chemical variables served as dependent variables in a factorial analysis-of-variance (ANOVA; 1998 fruit TGA data) or multiple analysis-of-variance (MANOVA; 1999 fruit chemical data), with the independent variables

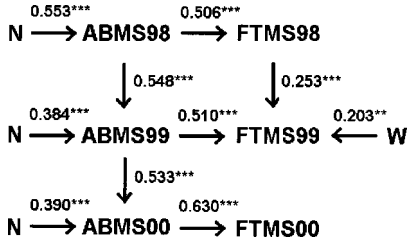

FIG. 1. Path analysis results showing effects of plant treatment on growth and reproduction across three growing seasons. Only statistically significant ($^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$) standardized regression coefficients are illustrated. N = Nitrogen treatment; W = Water treatment; ABMS98, ABMS99, ABMS00 = Aboveground dry mass (g) in 1998, 1999, and 2000, respectively; FTMS98, FTMS99, and FTMS00 = Total fruit wet mass (g) in 1998, 1999, and 2000, respectively.

being plant treatment and maternal plant. Because the overall MANOVA for the 1999 data was significant, we analyzed each of the dependent variables separately using univariate factorial analysis-of-variance (ANOVA), followed by post-hoc Tukey (HSD) comparisons of treatment means. These analyses were intended to determine which, if any, of the fruit pulp chemical parameters were affected significantly by treatment.

We calculated the Pearson correlations between leaf and fruit pulp PROT, PHEN, and TGA (for 1999 only) to determine if any of the variation in fruit chemistry could be attributed to variation in leaf chemistry, independent of the effects of treatment and maternal plant. Thus, this analysis tested the principal prediction of the PC hypothesis. We also calculated the Pearson correlation between ripe fruit TGA in 1998 with ripe fruit TGA in 1999 for those five maternal plants that produced ripe fruits in both years.

As another approach to determining the effect of leaf chemistry on fruit pulp chemistry, we included leaf chemical variables as covariates in a series of analyses-of-covariance. In each analysis, plant treatment and maternal plant served as the independent variables, a specific fruit chemical variable (e.g., fruit pulp TGA) acted as the dependent variable, and the corresponding leaf chemical variable (e.g., leaf TGA) served as the covariate. Significant covariate effects may be interpreted as evidence of physiologic constraints.

RESULTS

Effect of Plant Treatment on Growth and Reproduction. Path analysis results (Figure 1) were dominated by strong direct and indirect effects of N treatment on plant size (aboveground dry biomass), and by strong direct effects of plant size on fruit number and total fruit mass. The N treatments produced an approximate doubling of plant size, fruit number, and total fruit mass in each of the three growing seasons (Figure 2), although individual fruit mass was relatively unaffected by treatment in 2 of the 3 years. Water had little effect on growth and reproduction, and the effect of plant size on reproductive output suggested an allometric effect. In other words, increased N-based resources were apparently allocated proportionately to increased growth and reproduction. In any case, these results show a strong effect of N treatment on plant growth and reproduction, including the year that fruit and leaf samples were taken for analysis. These results were necessary to demonstrate strong effects of N treatment that must be present if effects on fruit chemistry are to be expected.

Fruit Chemistry as a Function of Plant Treatment. Despite the strong effects of plant treatment on growth and reproduction, we found only minor effects on fruit chemistry. In 1998, ripe fruit TGA varied little across treatments and among maternal plants (Table 2A and B). ANOVA results were not significant

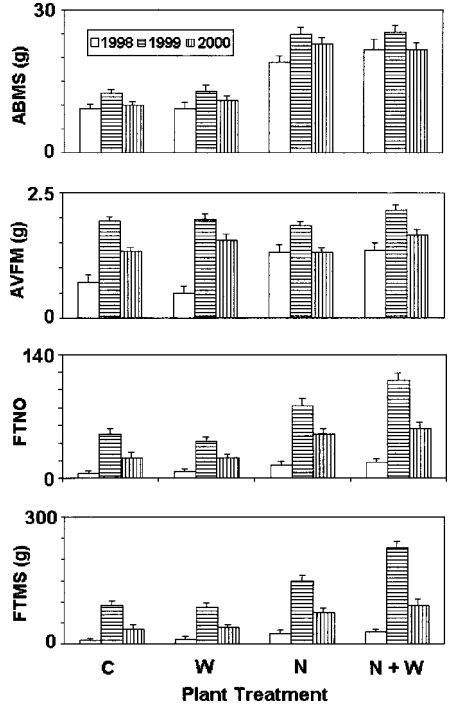

FIG. 2. Growth and reproductive data by plant treatment. Error bars represent standard errors of the mean across all maternal plants. Dependent variables include: total aboveground dry biomass (ABMS), average wet fruit mass (AVFM), total fruit number (FTNO), and total wet fruit mass (FTMS). Treatments are control (C), water (W), nitrogen (N), and nitrogen plus water (N + W).

for either factor (Table 2C). In 1999, means for PROT, PHEN, TGA, and SUG varied somewhat across treatments (Table 3A), as indicated by significant overall MANOVA effects for treatment and maternal plant (Table 3B). The significant MANOVA results could be explained by complex variation across treatments in PROT (N > C, W, and NW), PHEN (NW > C and W, N > W), and SUG (NW > N, W > N; Table 3A), and among maternal plants (where both PHEN and TGA were found to vary significantly; Table 3C). Most importantly, TGA did not vary significantly among treatments. Only one treatment by maternal plant interaction was found to be significant (for TGA), suggesting that the maternal plants generally responded similarly to the four treatments (Figure 3). Although treatment effects were statistically significant, these results were in contrast to our predictions (e.g., N-containing metabolites did not respond uniformly in a positive direction to N-supplementation). Furthermore, relative to the large differences in growth

TABLE 2. MEANS AND ANOVA RESULTS FOR EFFECTS OF PLANT TREATMENT[a] AND MATERNAL PLANT ON RIPE FRUIT TGA (1998 DATA)

	A. Means by plant treatment
Treatment	TGA (mg g^{-1} dry mass $\pm$ standard error; $N = 10$ per treatment)
C	13.42 ± 1.26
W	13.07 ± 1.43
N	14.93 ± 1.59
N + W	15.85 ± 1.14
	B. Means by maternal source
ID	TGA (mg g^{-1} dry mass $\pm$ standard error; $N = 8$ per clone)
AUG	11.77 ± 1.27
KENT	12.87 ± 1.43
N2O	14.21 ± 1.59
SACC	16.49 ± 1.14
TIFT	16.26 ± 1.24

C. ANOVA results; dependent variable = ripe fruit TGA

Effect	Hypothesis *df*	Error *df*	*F*	*P*
Intercept	1	4	240.737	<0.001
Treatment (T)	3	12	0.638	0.605
Maternal Plant (M)	4	12	1.294	0.327
T × M	12	20	2.335	0.045

[a] Treatment = control (C), water (W), nitrogen (N), and nitrogen plus water (N + W).

and reproduction produced by these plant treatments, the overall effect on fruit chemistry was small and unlikely to be biologically meaningful with respect to fruit defense and dispersal.

Correlation Between Leaf and Fruit Pulp Chemistry. The correlation between ripe fruit pulp TGA in 1998 and 1999 was significant for the plants producing fruits in both years; however, correlations between leaf and fruit pulp PROT, PHEN, and TGA were not significant in 1999, when all clones produced fruits (Table 4). These results do not support the PC hypothesis, which predicts strong correlations between leaf and fruit chemistry due to physiological effects.

ANCOVA Results for Effect of Leaf Chemistry on Fruit Pulp Chemistry. Supporting the results obtained from Pearson correlations, the results of ANCOVA analyses (Table 5) show no significant effects of leaf chemistry on ripe fruit pulp chemistry. In each case (PROT, PHEN, TGA), despite some significant treatment and maternal plant effects, the leaf chemical covariate was not a significant factor.

TABLE 3. MEANS AND MANOVA RESULTS FOR EFFECTS OF PLANT TREATMENT[a] AND MATERNAL PLANT ON RIPE FRUIT CHEMISTRY (1999 DATA)

	A. Means (mg g^{-1} dry mass $\pm$ standard error; $N = 30$ per treatment)[b]			
Treatment	PROT	PHEN	TGA	SUG
C	50.39 $\pm$ 5.12a	35.04 $\pm$ 2.37ab	16.05 $\pm$ 1.04a	181.3 $\pm$ 9.50ac
W	62.59 $\pm$ 8.58ab	32.00 $\pm$ 1.93a	14.39 $\pm$ 0.85a	183.2 $\pm$ 8.70ab
N	75.94 $\pm$ 4.91b	40.66 $\pm$ 1.89bc	17.53 $\pm$ 0.72a	166.1 $\pm$ 11.5c
NW	54.08 $\pm$ 6.43ab	44.12 $\pm$ 2.39c	15.75 $\pm$ 1.25a	210.4 $\pm$ 12.6b

B. MANOVA: Test of overall effects

Source	Pillai's trace	*F*	Hypothesis *df*	Error *df*	*P*
Intercept	0.983	1113.125	4	76	<0.001
Treatment	0.495	3.849	12	234	<0.001
Maternal Plant (M)	0.711	1.898	36	316	0.002
T $\times$ M	1.235	1.307	108	316	0.039

C. MANOVA: Tests of between-subjects effects

Source	Dependent variable	*df*	*f*	*P*
Intercept	PROT	1	341.35	<0.001
	PHEN	1	1408.32	<0.001
	TGA	1	1369.37	<0.001
	SUG	1	1239.86	<0.001
Treatment (T)	PROT	3	2.98	0.036
	PHEN	3	7.52	<0.001
	TGA	3	2.62	0.057
	SUG	3	2.86	0.042
Maternal Plant (M)	PROT	9	0.47	0.889
	PHEN	9	2.03	0.047
	TGA	9	5.13	<0.001
	SUG	9	0.92	0.516
T $\times$ M	PROT	1	1.00	0.478
	PHEN	1	1.28	0.199
	TGA	1	1.76	0.028
	SUG	1	1.29	0.193

[a] Treatment = control (C), water (W), nitrogen (N), and nitrogen plus water (N + W).
[b] Identical letters denote means that did not differ significantly ($P > 0.05$) based upon Tukey (HSD) post-hoc means comparisons following one-way ANOVA.

DISCUSSION

This study was designed to address the effect of plant nutrient variation on growth, reproduction, and fruit chemistry within a fleshy-fruited perennial plant.

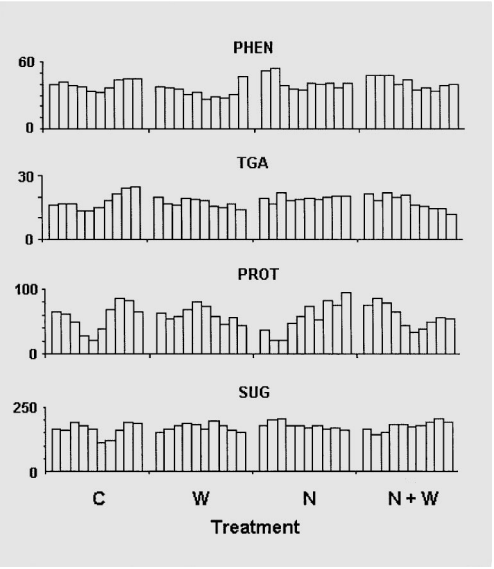

FIG. 3. Fruit chemical data (1999) by plant treatment and maternal plant source (clone). Within each treatment, vertical bars are ordered by maternal plant (AUG, EAST, HOW, KENT, N2O, SACC, SEN, SERC, TIFT, TWIN). Treatments are control (C), water (W), nitrogen (N), and nitrogen plus water (N + W). All values are mg g^{-1} dry mass; error bars are omitted for clarity.

While path analysis demonstrated that growth and reproduction was strongly affected by N availability, results concerning fruit and leaf chemistry did not support supply-side hypotheses. Rather, fruit chemistry remained relatively unaffected despite a widely varying total allocation of resources to fruits (fruit number, individual fruit mass, total fruit mass). We found no evidence that leaf and ripe fruit TGA are correlated, indicating that this species (*S. carolinense*), at least, is freely able to vary levels within each tissue type, which is at variance with the PC hypothesis in particular.

TABLE 4. PEARSON CORRELATIONS BETWEEN LEAF AND FRUIT CHEMICAL VARIABLES ACROSS ALL TREATMENTS AND MATERNAL PLANTS

Correlation	*N*	*R*	*P*
Fruit TGA 1998 and Fruit TGA 1999	40	0.381	0.015
Leaf PROT 1999 and Fruit PROT 1999	120	−0.153	0.096
Leaf PHEN 1999 and Fruit PHEN 1999	120	0.136	0.140
Leaf TGA 1999 and Fruit TGA 1999	120	−0.013	0.890

TABLE 5. ANCOVA ANALYSES FOR THE EFFECT OF PLANT TREATMENT[a], MATERNAL PLANT, AND LEAF CHEMISTRY ON RIPE FRUIT CHEMISTRY

Effect	*df*	*F*	*P*
A. ANCOVA results; dependent variable = ripe fruit PROT, covariate = leaf PROT			
Intercept	1	37.911	<0.001
Treatment (T)	3	2.912	0.040
Maternal Plant (M)	9	0.502	0.869
T × M	27	0.929	0.571
Covariate	1	1.124	0.292
Error	79	—	—
B. ANCOVA results; dependent variable = ripe fruit PHEN; covariate = leaf PHEN			
Intercept	1	380.971	<0.001
Treatment (T)	3	7.026	<0.001
Maternal Plant (M)	9	2.124	0.037
T × M	27	1.303	0.183
Covariate	1	0.295	0.589
Error	79	—	—
C. ANCOVA results; dependent variable = ripe fruit TGA; covariate = leaf TGA			
Intercept	1	517.611	<0.001
Treatment (T)	3	2.408	0.073
Maternal Plant (M)	9	5.496	<0.001
T × M	27	1.853	0.018
Covariate	1	0.436	0.511
Error	79	—	—

[a] Treatment = control (C), water (W), nitrogen (N), and nitrogen plus water (N + W).

To our knowledge, supply-side hypotheses such as the PC hypothesis have never been tested focusing on fruits within a plant species. Many recent tests of supply-side hypotheses focusing on foliage or whole plant materials have met with equivocal results, leading some to conclude that such hypotheses offer little predictive value (e.g., Hamilton et al., 2001). Our results support this general conclusion. In a phylogenetically-corrected, interspecific test of the PC hypothesis focusing on fruits of *Solanum* species, Cipollini et al. (2002a) found a positive relationship between unripe and ripe fruit TGA (a relationship not addressed in our present study). However, like the current study, no relationship of leaf TGA to either unripe or ripe fruit TGA was found in the interspecific study. A recalculation of the reported relationship between unripe and ripe fruit chemistry using a larger number of species supports the preliminary conclusion ($N = 48$ species, $r = 0.5181$, $P < 0.01$; Cipollini et al. unpublished data). The PC hypothesis predicts such a correlation if physiology constrains the removal of toxins from ripe fruits within species; those species with high levels in unripe fruits (for whatever reason) should also have high levels in the ripe fruits (as a consequence of physiology). Nevertheless, there are a number of species with high levels of TGA

in unripe fruit that produce low or nondetectable levels in ripe fruit, suggesting that any constraint that unripe fruit chemistry might place on ripe fruit chemistry must be weak or nonexistent in some species. Furthermore, the data in Cipollini et al. (2002a) suggest that *Solanum* species never start out with low levels of TGA in unripe fruits and then increase the concentration as fruits ripen. One interpretation of these results is that TGA may play similar functional roles (e.g., defense from pathogens) in both the unripe and ripe fruits. For certain species, that functional role may be important throughout the ripening phase. Many examples exist of *Solanum* species with minimal levels of glycoalkaloids in the leaves, and high levels in both unripe and ripe fruits (our present study species included).

The hypothesis most consistent with our results is the Defense Tradeoff hypothesis (Cipollini and Levey, 1997b), which is an OD-type hypothesis. Although some of the assumptions of the OD hypothesis are difficult to quantify for fruits (e.g., the long-term fitness value of fruit pulp, the fitness value of allocation to various secondary metabolites, the "costs" of secondary metabolites), and constraints exist which make OD predictions complex (Berenbaum and Zangerl, 1988; Linhart, 1991), it is a hypothesis grounded firmly in evolutionary theory. In its most general form, this hypothesis states that plants should allocate resources to secondary metabolites in ways that maximize fitness—the exact ways will depend upon the biology, history, and ecology of the system being studied. With reference to our study, we suggest that the particular balance of primary (nutrient) and secondary (toxic, deterrent) metabolites in the ripe fruit is optimized (insofar as possible) between attractive and defensive functions. If so, increased available N might be expected to increase plant size and/or growth rate, and consequently increase fruit number, but fruits should not vary strongly in individual "quality" (size, seed number, nutritive value, secondary metabolite content). This is the pattern that we found.

Results for the effect of plant treatment on leaf chemistry in this same set of plants showed a similar result generally supporting OD theory (Cipollini et al., 2002b). While N-treated (N, NW) plants in the year 2000 were twice as large and produced twice as many fruits as plants in the C and W treatments, they produced levels of leaf defense compounds and exhibited levels of insect herbivory similar to plants in low-N treatments. It is possible that the maintenance of antiherbivore defense in plants in the C- and W-treatment groups came at the expense of growth and fitness. In a similar manner, maintenance of fruit quality under low-N treatments may have come at the expense of lower fruit production.

While not a primary focus of this study, the relative lack of variation among maternal plants was surprising, given the wide area from which they were originally derived. The effect of maternal plant was involved in only a few significant interactions in our statistical analyses, indicating that the maternal plants generally responded similarly to treatments. Agricultural weeds that are experiencing

a relatively recent range expansion through North America have been shown to exhibit low levels of within-population genetic variation, although differences among populations can be significant (e.g., Warwick, 1990). It is possible that *S. carolinense* has shown such a recent range expansion. In addition to the potential bottleneck effects of recent range expansion, it is possible that low levels of interpopulation variation may result from the dispersal of clonal material through human activities, and relatively infrequent colonization of new habitats via sexual reproduction. Regardless, the results of this study provide insight into the general effects of resource variation on growth, reproduction, and fruit pulp chemistry in this species. In support of the Defense Tradeoff hypothesis, plants maintained fruit quality within a relatively narrow range despite large effects of nutrient variation upon growth and reproduction; the implication is that there is something "important" about that particular combination of fruit pulp traits.

With respect to the natural history of *S. carolinense*, it is likely that fruit pulp glycoalkaloids provide protection against insect and fungal pests during the prolonged periods of fruit ripening and persistence in the field. Fruits generally ripen on senescent stems during the midfall through winter in the southeastern United States and are relatively low in nutritional value. The fruits ripen at times when frugivore activities are reduced, particularly for the small mammals (foxes, raccoons, opossums) suspected of being the most common dispersers of the fruits. Thus, the retention of relatively high levels of defensive compounds may represent a compromise between attraction of such organisms and defense against pests and pathogens. The level of TGA typically found within ripe fruits of this species has been demonstrated to be highly deterrent and/or toxic to dispersing and nondispersing animals, to fungal pathogens, and to generalist herbivorous insects (Tingey, 1984; van Gelder, 1990; Flanders et al., 1992; Cipollini and Levey, 1997a,c). The brightly colored (yellow) and odorous (to humans) fruits are nevertheless dispersed over long time periods by animals that likely never consume more than a few fruits at a time. The net result is the dispersal of the seeds by the next growing season, which is the obvious "goal" of any dispersal syndrome.

While nonadaptive hypotheses explaining physical and nutritional patterns in fleshy fruits have gained some support (e.g., Bremer and Eriksson, 1992; Herrera, 1992; Jordano, 1995), our results suggest that framing future studies in terms of OD-type hypotheses might remain a productive and insightful approach for the study of secondary metabolites in fleshy fruits. Such hypotheses may be particularly useful in explaining the presence of substances in ripe fruits that are toxic or deterrent to dispersers, and seem to represent a paradox. Because these substances can *strongly* affect feeding behavior of frugivores and because they are likely important for fruit defense from pests and pathogens, they are more likely to be modified and maintained by selective pressures than the simple morphological or nutritional traits that are most commonly studied by fruit-frugivore researchers. Nevertheless, OD-type hypotheses may help explain allocation patterns of other

types of fruit metabolites as well. A simple example is that of fruit pigments that may attract not only legitimate seed dispersers, but also fruit pulp thieves and seed predators. The allocation of pigments in ripening fruits clearly involves a tradeoff between attraction and crypsis, and not all fruits change color significantly when ripening. The Defense-Tradeoff hypothesis would suggest that fruits that do not change color significantly have an advantage in that fruits are more difficult for pests to locate. Legitimate dispersers of such fruits should nonetheless be able to locate the fruits via other mechanisms (e.g., olfactory cues). Because OD-type hypotheses are not restricted in their application to a set of unusual plants with "toxic" fruits, we encourage the use of these concepts in framing future studies of secondary metabolites in ripe fruits of vertebrate-dispersed plants.

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JENSENONE: BIOLOGICAL REACTIVITY OF A MARSUPIAL ANTIFEEDANT FROM *Eucalyptus*

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Abstract—The resistance of *Eucalyptus* to browsing mammals has been related to the level and type of formylated phloroglucinol compounds (FPCs) present in the leaf. The antifeedant activity of FPCs appears to depend on their aldehyde groups, but little else is known of their mode of action. We have sought to elucidate this further by examining the biological reactivity and disposition of jensenone, a model FPC. Neither jensenone nor any metabolites were detected in urine or feces of marsupial brushtail or ringtail possums that had ingested up to 725 $mg \cdot kg^{-0.75}$. When jensenone was incubated in rat gastrointestinal segments *in vitro*, it rapidly disappeared. Jensenone also reacted rapidly with glutathione, cysteine, glycine, ethanolamine, and trypsin, and more slowly with acetylcysteine and albumin. Sideroxylonal, a more complex FPC, exhibited the same reactivity. Torquatone, a related compound that lacks both aldehyde groups and antifeedant activity, was unreactive. Mass spectroscopic analysis indicated that the adducts were Schiff bases formed between the aldehyde groups of FPCs and amine groups of the conjugating molecules. Successive adducts were formed with the two aldehyde groups of jensenone, and the four groups of sideroxylonal. The jensenone bis-glutathione adduct appeared to cyclize to the disulfide form. These findings suggest that the antifeedant effects of FPCs are due to their facile binding to amine groups on critical molecules in the gastrointestinal tract, leading to a loss of metabolic function. The consequent toxic reaction, probably involving chemical mediators such as 5-hydroxytryptamine (5HT), may cause colic, nausea, and a general malaise, resulting in anorexia.

Key Words—Antifeedant, plant secondary metabolite, *Eucalyptus*, jensenone, sideroxylonal, formyl phloroglucinols, glutathione conjugate, aldehyde.

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INTRODUCTION

Secondary compounds are ubiquitous in the diets of browsing mammals and there is much evidence that they can restrict the types and amount of plants eaten (Foley et al., 1999). For most ecologically important plant secondary metabolites (PSMs), we have little appreciation of the tissue targets that are affected by the PSM or of the pathways of detoxification and elimination of these compounds in consumers (Foley et al., 1999; McLean et al., 2001). If we are able to understand how PSMs are metabolized, we will better appreciate how variability in both plant and animal responses allow for the evolution of complex interactions between plants and herbivores. This is our objective in studies of *Eucalyptus* and its vertebrate herbivores in Australia.

Recent studies (Lawler et al., 1998a; Stapley et al., 2000) have demonstrated that the major factor that limits the amount of *Eucalyptus* leaves eaten by folivorous marsupials (e.g., koala, common ringtail, and common brushtail possum) is the concentration of formylated phloroglucinol compounds (FPCs) e.g., jensenone, sideroxylonal A (Figure 1). FPCs are highly diverse, but characterized by at least

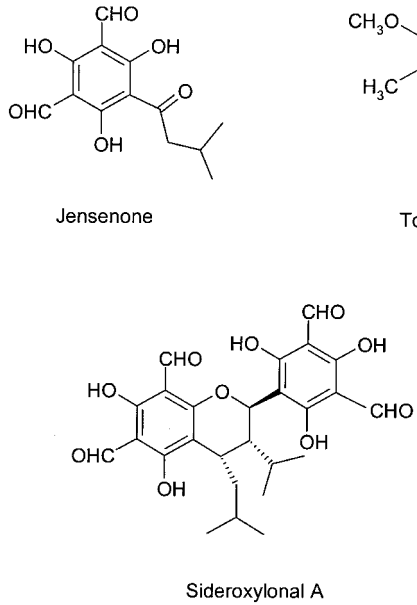

FIG. 1. Structures of phloroglucinol derivatives. Jensenone and sideroxylonal are formyl phloroglucinols (FPCs).

one fully substituted phenolic ring with one or two aldehyde groups, which are hydrogen bonded to the phenol group (Boland et al., 1992). Limited structure–function studies (Lawler et al., 1999a) have shown that the antifeedant effects of FPCs depend on the presence of the aldehyde group, but other correlational studies suggest other structural features are also important (B. D. Moore unpublished data).

Previous studies have shown that marsupials closely regulate their consumption of FPCs around a threshold, but that there is significant variation between species and among individual animals (Lawler et al., 1998a, 2000; Stapley et al., 2000). However, administration of the selective $5HT_3$-receptor antagonist, ondansetron (a powerful antiemetic), leads to significantly higher intakes of jensenone (Lawler et al., 1998b). This suggests that animals detect nauseous sensations from the ingestion of jensenone and so are able to titrate their intake in response to this. However, exactly how this occurs remains unknown.

In an attempt to understand why FPCs are such effective antifeedants and why some species and individual animals are more sensitive to their effects than others, we studied the biological disposition of jensenone in two species of marsupials, the common ringtail possum (*Pseudocheirus peregrinus*) and the common brushtail possum (*Trichosurus vulpecula*), by using a combination of *in vivo* and *in vitro* techniques. The findings led to an investigation of the biological reactivity of jensenone and its effects on the gastrointestinal tract.

METHODS AND MATERIALS

Animals. Details of the capture and care of brushtail (*Trichosurus vulpecula*) and ringtail possums (*Pseudocheirus peregrinus*) have been reported previously (Lawler et al., 1998a; Stapley et al., 2000). Rats (Hooded-Wistar) and guinea pigs were supplied by the Central Animal House, University of Tasmania. All animal procedures were approved by the Animal Experimentation Ethics Committee of the institution where each experiment was conducted.

Materials. Jensenone and torquatone were extracted and purified from *Eucalyptus jensenii* and *E. torquata* foliage, respectively (Lawler et al., 1999a). Sideroxylonal was extracted from *E. melliodora* foliage (Eschler and Foley, 1999). Extract of *Helix pomatia* (a mixture of β-glucuronidase 141,000 units/ml and aryl sulfatase 3950 units/ml) was obtained from Boehringer Mannheim (Germany) and *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) from Alltech Associates (Australia). Other chemicals and solvents were of analytical reagent grade. Trypsin and glutathione were from Boehringer Mannheim (Germany) and acetylcysteine, albumin and glycine from Sigma (Australia).

Jensenone Solutions. A standard solution of jensenone (50 mg in 2 ml 2% sodium carbonate) was prepared and diluted to give other concentrations. It had a

pH of 7–8 and was stable for at least 1 week by gas chromatographic (GC) analysis. Jensenone was readily extracted into ethyl acetate from aqueous solutions after acidification with 5 M HCl.

GC–FID. Extracts in ethyl acetate were dried under nitrogen and derivatized with BSTFA (20 μl, heated at 70°C for 5 min). GC analyses were conducted on a Varian 3300 instrument fitted with a 30 m Econocap capillary column (0.25 μm SE-54, 0.32 mm i.d.; Alltech Associates) and flame ionization detector (FID). GC conditions were: injection volume 1 μl, split ratio 1:20, carrier He at 9 psi, injector 250°C, detector 300°C, oven 150–290°C 10°C/min, held at 290°C for 9 min. Retention times of jensenone TMS derivatives were: major peak 12.2 min, minor peaks at 9.0, 9.4, and 13.4 min.

GC–MS. Details of the instrument and operating conditions for combined gas chromatography–mass spectrometry (GC–MS) have been reported previously (Boyle et al., 2000a). Jensenone gave a GC peak with a MW of 238 (rather than 266), corresponding to a degraded form.

LC–UV. Samples were either extracted into ethyl acetate, or simply filtered (0.45 μ) before analysis by liquid chromatography with UV detection (LC–UV). A Varian 9010 solvent delivery system with Rheodyne injector model 7161 was connected to a Varian 9050 UV–Vis absorbance detector and Star workstation (Varian Instruments, Melbourne). The column was a reverse-phase C18 Nova-Pak (3.9 $\times$ 150 mm; Waters Associates, Sydney), and the injection volume was 10 μl. The absorption maxima were: jensenone 268 nm; jensenone adducts 332 nm; sideroxylonal 280 nm; and torquatone 210 nm.

Several different mobile phase systems were required because of the variety of experimental procedures. System I was acetonitrile–1% acetic acid (85:15), 0.7 ml/min (jensenone eluted in 4.8 min). System II was methanol–1% acetic acid (55:45), 0.8 ml/min, programmed to 100% methanol at 15 min (jensenone eluted in 14.5 min). System III, used for sideroxylonal, was 2% acetic acid in methanol–2% acetic acid in water (96:4), 1 ml/min. Sideroxylonal exists in three isomeric forms (Eschler and Foley, 1999). It eluted as two peaks, at 2.29 min (sideroxylonal A/C) and 3.42 min (sideroxylonal B), and was quantitated by the sum of the two peaks.

LC–MS. Chromatography conditions for the analysis of jensenone reactions by liquid chromatography–mass spectrometry (LC–MS) were the same as LC–UV System II. Negative ion electrospray ionization (ESI) was used, and the instrument and operating conditions were as previously described (Boyle et al., 2001). MS/MS analyses were used to confirm the origin of product ions. Sideroxylonal reactions were analyzed by System IV: 2% acetic acid in methanol–2% acetic acid in water (80:20), programmed to 2% acetic acid in methanol in 10 min, 0.8 ml/min. Sideroxylonal peaks eluted at 7.58 min (A/C) and 10.02 min (B). MS of each peak produced ions at m/z 499 ($[M-H]^-$) and a fragment at m/z 249 (effectively half the molecule) (Neve et al., 1999).

Oral Dosing. Jensenone was incorporated into the feed of six brushtail and six ringtail possums, at concentrations of 0.0025–0.04% dry matter (Lawler et al., 1999b; Stapley et al., 2000). Intakes ranged from 428 to 725 $mg \cdot kg^{-0.75}$ (brushtails) and 8–48 $mg \cdot kg^{-0.75}$ (ringtails). In addition, two ringtail possums were gavaged with jensenone (dissolved in 2% sodium carbonate) at doses of 50 and 100 $mg \cdot kg^{-1}$, and two brushtails at a dose of 50 $mg \cdot kg^{-1}$. Urine and feces were collected for 24 hr and analyzed by LC and GC using methods similar to those used previously to characterize terpene metabolites (Boyle et al., 1999, 2000a,b, 2001). Jensenone was readily extracted from aqueous systems, although calibration curves were not prepared from excreta.

Disappearance of Jensenone from Rat Gastrointestinal Tissue. The rat gut tissue used was either the stomach or a segment (7–8 cm) of ileum, taken from a freshly-killed rat and washed and kept in Tyrode's solution (37°C, bubbled with air). One end of the tissue was ligated and the other end tied to a glass tube, from which the tissue was suspended in an organ bath. Jensenone (15 mg in 1 ml 1% sodium carbonate, pH 9) was placed in the tissue and samples of the contents for analysis were withdrawn through the glass tube at different times from 1 min onwards; the outer bath was also sampled. The gastrointestinal samples were centrifuged and filtered before analysis by LC–UV, or extracted into ethyl acetate and analyzed by GC–FID.

Attempts were made to recover jensenone from the gut tissue. After incubation with jensenone, segments of ileum were frozen in liquid nitrogen and ground with a mortar and pestle. Aliquots (100 mg) of the ground tissue and samples (50 μl) of the luminal contents were placed in a glass vial, 200 μl 5 M NaOH added, and the vial sealed and flushed with nitrogen. The contents were heated (60°C, 10 min), acidified, and extracted with ethyl acetate and analyzed for jensenone.

Reactivity of Jensenone. Jensenone (7.5 mg in 0.5 ml 2% sodium carbonate) was incubated at 37°C with 25 mg trypsin, albumin, glutathione, or acetylcysteine. Aliquots (10 or 100 μl) were taken, diluted, filtered, and analyzed by LC–UV. Experiments were conducted in duplicate and half-life calculated from the slope of the disappearance curve.

Formation of Adducts. Glutathione (25 mg) and jensenone (7.5 mg) were each dissolved in 0.5 ml 1% sodium carbonate and the solutions mixed to start the reaction. Aliquots (100 μl) were taken at different times, acidified with 2 drops 5 M HCl forming a precipitate that was removed by centrifugation and dissolved in methanol (2 ml). The methanol was filtered and analyzed by LC–UV and LC–MS. The temperature was lowered to 20°C in order to slow the reaction and follow product formation.

Glycine, L-cysteine, acetylcysteine, and ethanolamine were also tested for their ability to form adducts with jensenone. Jensenone (5 mg in 0.25 ml 1.0% sodium carbonate) was added to the test substance (15 mg in 0.25 ml 1.0% sodium carbonate) and incubated at 37°C for 60 min. Aliquots (10 μl) were removed

at different times, diluted with 0.1 ml distilled water and 0.9 ml methanol, and analyzed by LC–UV and LC–MS. Similarly, adduct formation was investigated by incubating glycine with sideroxylonal, torquatone or benzaldehyde.

Isolation of Adducts. Larger quantities of jensenone were reacted with glutathione, ethanolamine, or acetylcysteine, and the products acidified and extracted into ethyl acetate. Purification was by thin layer chromatography (silica gel) and crystallization (from ethyl acetate or methanol), as appropriate.

Rates of Reaction of Jensenone and Sideroxylonal with Glycine: Effect of pH. Because sideroxylonal is less soluble than jensenone in aqueous systems, lower concentrations and nonaqueous solvents were used to compare the reactivities of jensenone and sideroxylonal with glycine at different pH values. Two buffers were used: 0.067 M phosphate buffer for pH 7 and 8, and 0.1 M carbonate buffer for pH 9, 10, and 11. Jensenone (0.3 mg in 1.2 ml buffer) was added to 1.8 ml buffer containing 0.9 mg glycine and incubated at 25°C. Because of its insolubility, sideroxylonal was dissolved in ethyl acetate (1 mg/ml). Reactions were started by mixing 0. 3 ml (0.3 mg) of this solution with 0. 9 ml methanol, and adding it to 1.8 ml buffer containing 0.9 mg glycine and incubating as above. Methanol was omitted from the jensenone reaction because it caused LC peak broadening. Aliquots (100 μl) of each reaction mixture were removed at different times, mixed with methanol (200 μl), and analyzed by LC–UV. Sufficient 0.5 M HCl was included in the 200 μl methanol to neutralize the reaction mixture. As a check on stability, controls were run with glycine omitted.

Response of the Guinea Pig Ileum to Jensenone. A segment of ileum was taken from a guinea pig which had just been euthanized with pentobarbitone, and suspended in a 10 ml organ bath in Tyrode's solution at 37°C and gassed with air. Sublimed jensenone was dissolved in 0.5% sodium carbonate (10 mg $\cdot$ ml^{-1}) and diluted with buffer (pH 7). Histamine solutions were prepared in Tyrode's solution.

Effects of Jensenone on Histology of Rat Tissues. Hooded Wistar rats (male, 200–250 g) were starved overnight and gavaged the next morning with jensenone (10 mg in 1 ml 0.5% sodium carbonate, pH adjusted to 7–8) or vehicle (controls). The animals were euthanized (pentobarbitone) after 2, 6, and 24 hr, and tissues (stomach, duodenum, ileum, liver, kidney) taken for examination and placed in buffered formalin. Tissues were stained with haematoxylin and eosin before examination by light microscopy.

RESULTS

Metabolic Fate of Jensenone In Vivo. In several experiments, no trace of jensenone or related products was detected in the urine or feces of possums that had ingested jensenone. Excreta were extracted (before and after incubation with β-glucuronidase/arylsulfatase) and analyzed by GC–FID, GC–MS, and LC–UV.

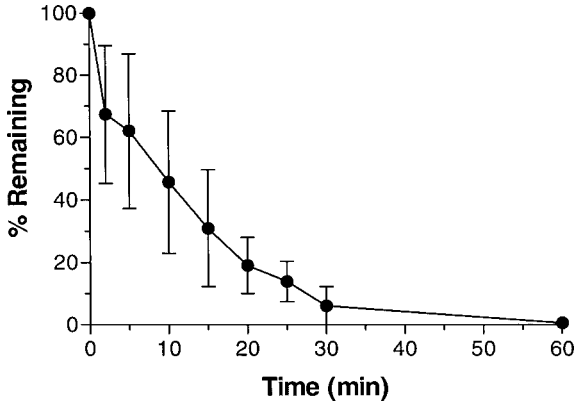

FIG. 2. Disappearance of jensenone from rat ileum (mean ± SE, $N = 3$).

Comparison with excreta from undosed animals showed that there were no new peaks in chromatograms from animals that had ingested jensenone.

Disappearance of Jensenone from Rat Gastrointestinal Tissue In Vitro. Having failed to find evidence of the excretion of jensenone or its metabolites, the fate of jensenone was investigated in isolated segments of rat ileum. The gut responded with vigorous activity and the contents became thick with mucus. Jensenone disappeared rapidly from the lumen (Figure 2), although none was detected in the outer bath. In similar experiments, jensenone also rapidly disappeared from the isolated rat stomach. After the disappearance of jensenone, variable amounts (8–33%) could be recovered after heating samples of tissue with sodium hydroxide.

Reactivity of Jensenone. Jensenone was stable in 2% sodium carbonate solution, but disappeared slowly when stored in methanol. In order to explore the possible causes of the disappearance of jensenone from gastrointestinal segments, it was incubated with various endogenous molecules: a gastrointestinal enzyme (trypsin), a protein (albumin), and two nucleophilic thiols (glutathione and acetylcysteine) known to protect cells against reactive electrophiles (De Vries and De Flora, 1993; Dickinson and Forman, 2002). Figure 3 shows that jensenone disappeared rapidly when incubated with trypsin (half life, $t_{1/2} = 5.4$ min) or glutathione ($t_{1/2} = 2.6$ min), and more slowly with albumin ($t_{1/2} = 91$ min) or acetylcysteine ($t_{1/2} = 69$ min).

Formation of Adducts. The reaction of glutathione and jensenone was analyzed after 60 min by LC–MS (Figure 4A). The monoadduct of glutathione and jensenone eluted at 6.9 min. It was characterized by the following ions: m/z 554 ($[M-H]^-$), 510 ($[M-H-CO_2]^-$), 476 ($[M-H-CO_2-H_2S]^-$), and sodium adducts m/z 576 and 598 (Figure 4B). The loss of H_2S indicated that the thiol

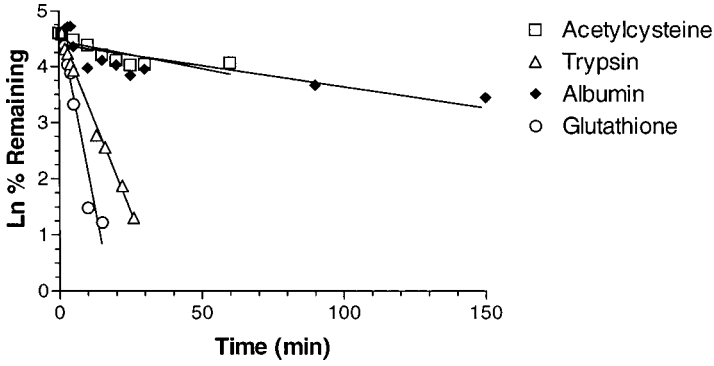

FIG. 3. Disappearance of jensenone when incubated with various compounds.

group was not involved in the bond between jensenone and glutathione. There was also a bis-adduct (jensenone and two glutathiones), which eluted at 3.01 min and showed characteristic ions in the same pattern. These were: m/z 843 ($[M-H]^-$), 799 ($[M-H-CO_2]^-$), 755 ($[M-H-2CO_2]^-$), and 721 ($[M-H-2CO_2-H_2S]^-$). However, the major LC peak (eluting at 2.68 min) had a molecular weight two daltons less than this and was interpreted as the bis-adduct with an internal disulfide bond, formed by oxidative linkage of the two glutathione sulphur atoms (Figure 5). It gave ions analogous to the bis-adduct following the loss of two protons: m/z 841 ($[M-H]^-$) and sequential losses of CO_2 to give ions at m/z 797 and 753, followed by loss of H_2S to give an ion at m/z 719 (Figure 4C). Figure 6 shows the sequential formation of mono- and bis-adducts of glutathione and jensenone. The presence of methanol resulted in the formation of methyl esters of the glutathione carboxylate groups, which were identified by MS.

Jensenone readily reacted with L-cysteine, glycine, and ethanolamine, in each case giving one major product by LC–MS, with the general formula: (jensenone + 2 amine $-2H_2O$). With acetylcysteine, jensenone produced one major product with an elution time of 2.56 min (LC System I). The negative ion electrospray mass spectrum gave an $[M-H]^-$ion at m/z 237 corresponding to a molecule consistent with (jensenone − CO).

Glycine readily reacted with sideroxylonal. The sideroxylonal–monoglycine adduct (MW 557) produced an ion at m/z 556 ($[M-H]^-$) and fragments at m/z 249 (without glycine) and 305 (i.e., m/z 249 with glycine). Sideroxylonal has four aldehyde groups, and there was clear evidence of sideroxylonal linking to two and three glycines, producing $[M-H]^-$ ions at m/z 613 and 670, respectively.

There was no reaction when benzaldehyde was incubated with glycine at 37°C (pH 7 or 8) for 24 hr. There was also no reaction of glycine with torquatone, a phloroglucinol derivative that lacks aldehyde groups (Figure 1).

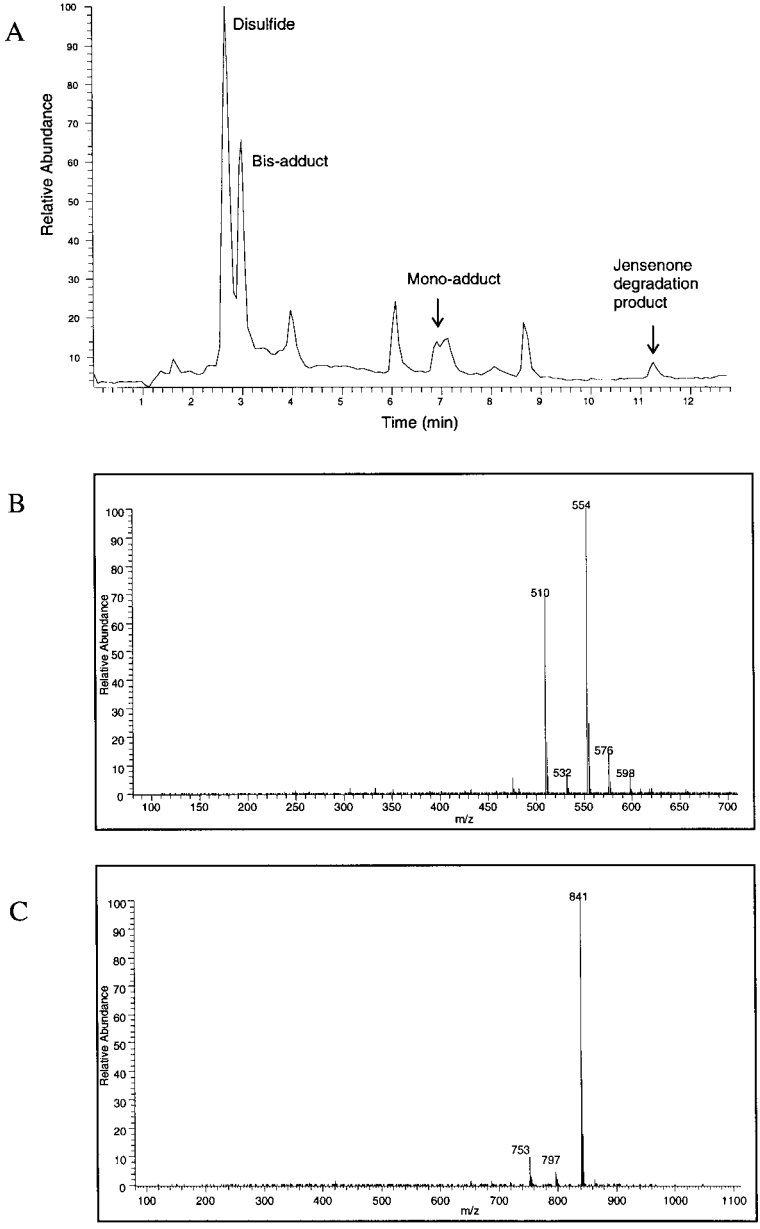

FIG. 4. (A) Negative ion ESI LC-MS chromatogram of glutathione adducts of jensenone. (B) Mass spectrum of mono-adduct (6.9 min). (C) Mass spectrum of bis-glutathione adduct with disulfide bridge (2.7 min).

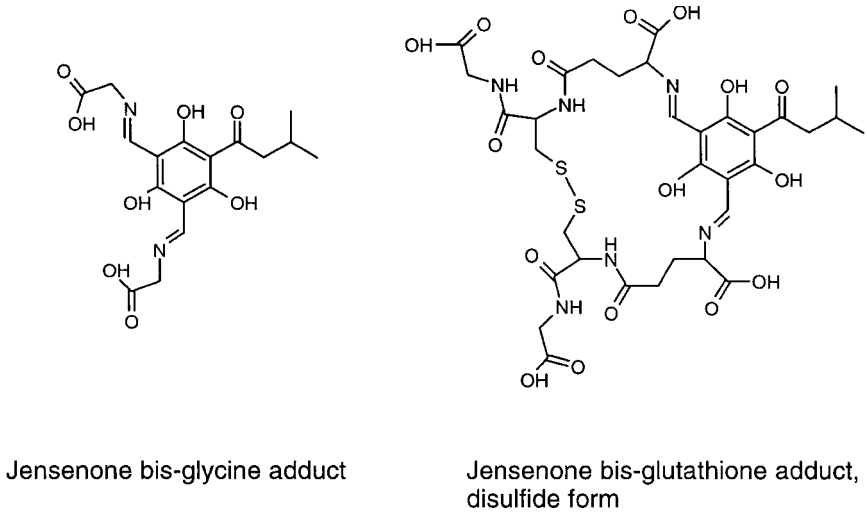

FIG. 5. Proposed structures of jensenone adducts with glycine and glutathione.

Isolation of Adducts. Attempts were made to prepare sufficient quantities of the adducts to enable NMR analysis. This was attempted with the adducts formed with glutathione and ethanolamine. However, these jensenone derivatives proved to be unstable during workup and storage. Also, the NMR data obtained indicated that the derivatives existed in two interchangable forms, resulting in a lack of a clear NMR spectrum. As this interchangability probably involves the phenolic protons that can hydrogen-bond with the carbonyl oxygens in two forms (as in jensenone

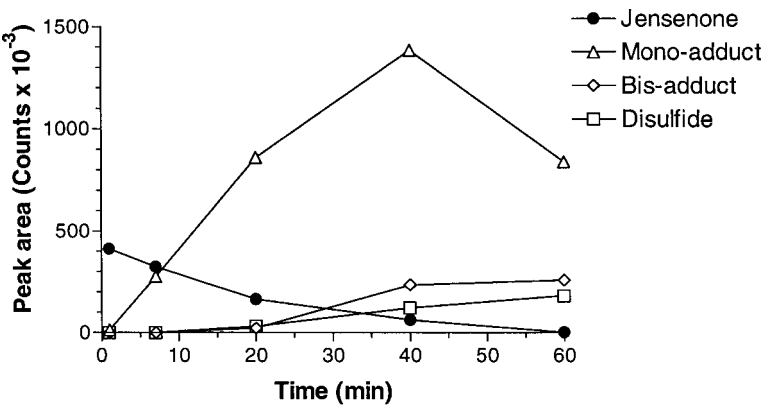

FIG. 6. Rates of formation of adducts of jensenone and glutathione.

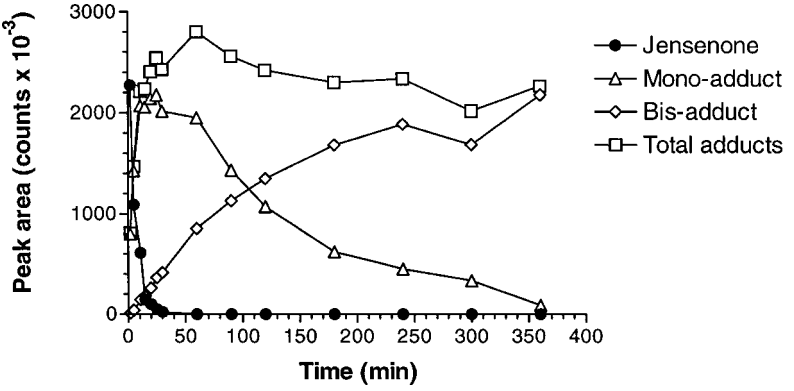

FIG. 7. Reaction of jensenone with glycine at pH 8.

itself; Boland et al., 1992; Ghisalberti, 1996), attempts were made to remove them by methylation, but this resulted in the methyl esters of the glutathione portion. Acetylation of the phenolic groups was also attempted, but this gave rise to multiple products.

Attempts were also made to prepare and characterize the product of acetylcysteine and jensenone. However, it was an orange oil that did not give a clear NMR signal.

Rates of Reaction of Jensenone and Sideroxylonal with Glycine: Effect of pH. The reactivity of jensenone and sideroxylonal were compared at different pH values, using glycine as a simple biological nucleophile. As an example, the reaction of jensenone and glycine at pH 8 is shown in Figure 7. The sequential formation of mono- and bis-adducts followed the disappearance of jensenone. The glycine adducts of sideroxylonal did not resolve by LC so the reaction was followed as the disappearance of sideroxylonal. Control incubations in buffer without glycine showed that there was no significant loss of jensenone or sideroxylonal during the incubation period.

Jensenone and sideroxylonal reacted with glycine over the pH range 7–11, and reacted faster at higher pH values. The half-life of jensenone fell from 234 min at pH 7 to 33 min at pH 11, while the corresponding half-lives for sideroxylonal were 140 min and 41 min, respectively. In each case, a plot of ln (half-life) versus pH gave a straight line: jensenone slope -0.521 ± 0.056 (mean $\pm$ SD, $N = 5$), $r^2 = 0.967$ and sideroxylonal slope -0.291 ± 0.026, $r^2 = 0.967$. These slopes were significantly different ($P < 0.01$, ANCOVA).

Response of the Guinea Pig Ileum to Jensenone. Because of the increased motor activity observed in the rat gut segments containing jensenone, the contractile response to jensenone was studied by using the standard guinea pig ileum preparation.

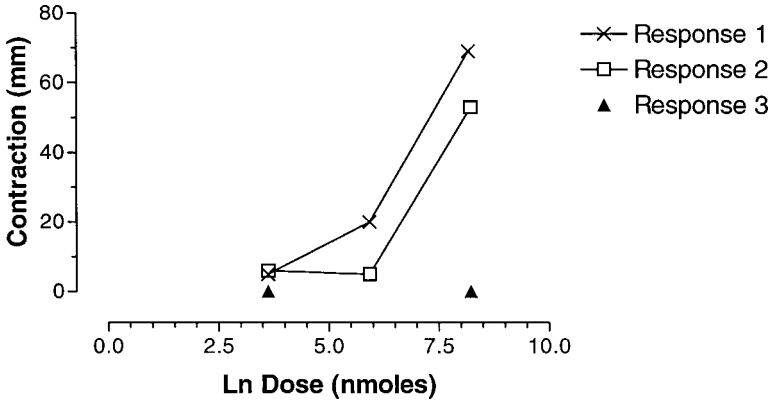

FIG. 8. Response of the guinea pig ileum to three doses of jensenone. The low dose (38 nmoles) was given three times, the middle dose (376 nmoles) was given twice, then the high dose (3760 nmoles) was given three times.

The ileum contacted when jensenone was injected into the bath water, but high concentrations were required (3.8 μmoles in the 10 ml organ bath). The same maximal contraction was produced by only 1.0 nmole histamine. The response to a dose of jensenone was not sustained (i.e., the contraction diminished with time) whereas the histamine response stayed constant until the tissue was washed. Repeated dosing with jensenone resulted in a diminishing response (Figure 8). In another segment of ileum, repeated administration of the high dose (1 mg, 3760 nmoles) produced a response that fell progressively from a maximum to zero in four repeats. The response to histamine was also decreased by jensenone treatment (data not shown).

The rapidly-decreasing response to jensenone made it difficult to assess the effect of possible antagonists. However, there was no evidence that a large dose of atropine (100 nmoles; sufficient to abolish the maximum response elicited by histamine, 1.0 nmoles) decreased the response to jensenone.

Effects of Jensenone on Histology of Rat Tissues. In comparison with controls, the jensenone-dosed rats lay quietly, flat on the stomach, and tended to have a wet muzzle, evidently due to salivation. These effects developed soon after the treatment, but the animals had recovered normal behavior by 6 hr. Upon opening the abdomen after euthanasia at 2 and 6 hr, the stomach and small intestine appeared distended by the jensenone dose. Tissue slides indicated some vacuolation at the tip of the villi, possible loss of villi and sloughing of cells. However, these findings were equivocal, and all other tissues appeared normal.

DISCUSSION

The initial objective of this study was to describe the metabolic fate of jensenone, a plant secondary metabolite (PSM) with a powerful antifeedant effect. Jensenone was considered to be a model for other antifeedant FPCs such as sideroxylonal. We have previously used the same general analytical methodology to describe the metabolites of terpene and phenolic PSMs in marsupial folivores (Boyle et al., 1999, 2000a,b, 2001; McLean et al., 2001, 2003). However, despite a thorough investigation, no evidence of jensenone or related compounds was found in the urine or feces of brushtail or ringtail possums that had ingested large amounts of jensenone. Either the jensenone was so chemically changed that it was unrecognizable, or it was sequestered at tissue sites. Incubation of jensenone in isolated segments of rat gastrointestinal tissue showed that it rapidly disappeared, indicating that there was a chemical reaction between jensenone and tissue sites.

Jensenone possesses two aldehyde and three phenolic groups. Aldehydes are reactive electrophilic molecules that undergo nucleophilic addition reactions (March, 1992) and are known to form covalent adducts with biological molecules. For example, the toxicity of ethanol is in part mediated by its metabolite, acetaldehyde, which forms adducts with proteins, leading to tissue injury (Lieber, 1991). Several potential target molecules were examined for their reaction with jensenone. The pancreatic enzyme, trypsin, is a protein that ingested jensenone would encounter in the small intestine. Albumin was assessed as another protein and one that jensenone would come into contact with if it were absorbed into the bloodstream. Both these proteins reacted with jensenone, albeit slowly in the case of albumin. Reaction with these proteins cannot explain the rapid disappearance of jensenone from the *in vitro* gut system, where they are absent, although other proteins could be involved. Smaller biological nucleophiles were then examined to elucidate the chemistry of jensenone reactivity.

Glutathione is a tripeptide that reacts with an enormous array of electrophilic xenobiotics to protect cellular nucleophiles (proteins and nucleic acids) from attack by toxic molecules (Parkinson, 2001). Electrophiles generally bind to the thiol group of reduced glutathione giving rise to a thio-adduct that can be subsequently converted into a mercapturic acid or other thioether metabolites (Parkinson, 2001). Although the initial reaction is catalyzed by a family of glutathione-*S*-transferases that are present at high levels, in the gut, liver, and other tissues, the electrophilic substrates typically also react nonenzymatically with glutathione (Ketterer, 1982, 1986). Acetylcysteine is another thiol nucleophile that undergoes similar reactions to glutathione and is used therapeutically to protect against electrophilic toxicity, most notably in the case of paracetamol overdose (Forrest et al., 1982; De Vries and De Flora, 1993). Jensenone reacted very rapidly with glutathione, and this was

initially interpreted as thioadduct formation. Jensenone reacted readily with the thio-amino acid cysteine that like glutathione, has both a thiol and amino group free. However, jensenone reacted much more slowly with acetylcysteine. The product of this reaction, equivalent to (jensenone − CO), was not analogous to the adducts formed with other nucleophiles. Acetylcysteine has a free thiol group, but the amine is blocked by acetylation. This was the first indication that the reaction with jensenone may involve amines rather than thiols, and was confirmed by the observation that jensenone reacted rapidly with the thiol-free amines, glycine, and ethanolamine.

MS analyses were consistent with the sequential formation of Schiff base adducts on each of the aldehyde groups of jensenone and sideroxylonal. There was MS evidence that the bis-glutathione–jensenone adduct cyclized to form the intramolecular disulfide, which also indicated that the glutathione thiols remained free after adduct formation.

Although aldehydes can react with thiols to form thiohemiacetals, they are also able to undergo addition reactions with amines to form a Schiff base (March, 1992). Schiff bases are most stable when conjugated with an aromatic group, but they also occur between nonaromatic biological molecules, for example acetaldehyde and lysine (Braun et al., 1995). On the other hand, glutathione reportedly forms a hemithioacetal with acetaldehyde (Ketterer, 1982), dichloroacetaldehyde (Guengerich and Liebler, 1985), and formaldehyde (Mason et al., 1986; Naylor et al., 1988). More often, though, the carbonyl group activates an adjacent double bond leading to a Michael addition reaction and thioether formation (Parkinson, 2001). This can occur at an aromatic ring, as in the reactive metabolite of paracetamol, *N*-acetylbenzoquinoneimine. We found no nonenzymatic reaction between benzaldehyde and glycine, although there is evidence that rat liver preparations can catalyze the formation of the *S*-benzyl glutathione conjugate from benzaldehyde (Mutlib et al., 2002).

The two aldehyde groups of ortho-phthalaldehyde react under alkaline conditions with both the amine and thiol groups of glutathione to form a cyclic derivative, which can be used to quantitate glutathione (Neuschwander-Tetri and Roll, 1989). An extensive search of the literature did not reveal any other reports of Schiff base formation with glutathione.

The sesquiterpene 1,4-dialdehyde polygodial reacts rapidly with cysteine, and its antifeedant properties, and hot taste, have been attributed to interactions between its aldehyde groups and tissue thiols (Kubo and Ganjian, 1981). However, analysis of the product of this reaction indicated that it first formed a Schiff base between an aldehyde group and the cysteine amine, followed by addition of the cysteine thiol group to the double bond, giving a thiazolidine ring (D'Ischia et al., 1982). This is a known reaction of aldehydes with cysteine (Jocelyn, 1972).

The question of why jensenone reacts preferentially with the amine rather than the thiol group of glutathione may be explained by the hard–soft theory

of reactivity (Guengerich and Liebler, 1985; Ketterer, 1986). Hard electrophiles (or nucleophiles) have a dense localization of charge, whereas the soft forms have a low charge density that is readily polarizable. Thiols are soft nucleophiles because of the large atomic volume of sulphur, whereas amines are relatively hard nucleophiles. Carbonyls are hard electrophiles, which react more readily with hard nucleophiles, while soft nucleophiles react with soft electrophiles, in both cases because the energy of activation is lower (Ketterer, 1986).

The adduct formation was faster at higher pHs for jensenone and sideroxylonal, supporting a base-catalyzed mechanism, with the amine in the nonprotonated form (March, 1992). The reactions proceeded rapidly over the pH range tested (pH 7–11), indicating that adduct formation will occur at all pHs likely to be encountered in the mammalian intestine.

The chemical (and biological) activity of aldehydes varies greatly, even within a structurally-similar group such as the unsaturated 1,4-dialdehydes (Jonassohn and Sterner, 1997). In these experiments, we found that benzaldehyde did not react with glycine under the conditions where jensenone and sideroxylonal reacted rapidly and completely. On the other hand, the sesquiterpene 1,4-dialdehyde polygodial reacts readily with primary amines to form stable pyrrole derivatives (Brooks et al., 1989; Jonassohn and Sterner, 1997). Gossypol, an insect antifeedant compound found in cotton plants, is a sesquiterpene dimer with two aldehyde groups that readily form imines with the amino groups of proteins (Gershenzon and Croteau, 1991). It is proposed that gossypol acts by binding dietary protein (making it less digestible) and/or inhibiting digestive enzymes.

The relatively high dose of jensenone required for its biological effects suggests a nonspecific mechanism, involving reaction with amino groups on various biological molecules causing enzyme inhibition and toxicity. Inflammatory mediators such as 5HT have been implicated in the antifeedant effects of jensenone (Lawler et al., 1998b). However, some sesquiterpene unsaturated dialdehydes appear to activate sensory neurons by stimulation of vanilloid receptors (VR) (Szallasi and Blumberg, 1999). VR respond to painful chemical stimuli, such as capsaicin, leading to pain and inflammation. It is possible that at least part of the effects of jensenone are mediated via VR.

The contractile response of the guinea pig ileum did not appear to be mediated through acetylcholine or histamine receptors, since it was not sustained nor was it blocked by a large dose of atropine. In fact, the low potency of jensenone suggests a nonspecific mechanism, and tachyphylaxis suggests rapid depletion of component(s) necessary for the response. Since the histamine response was also diminished, this points to a general mechanism, possibly involving the depletion of enzymes or energy required for contraction.

Although rats appeared sick after an oral dose of jensenone, they had regained normal behavior by 6 hr, and there was no clear histological evidence of damage to the upper gastrointestinal tract. Therefore, although jensenone (and presumably

related FPCs) can covalently bind to tissue amines, its effects were not irreversible, at least at the dose assessed.

Reactive electrophiles can form adducts with proteins via either of the two major nucleophilic groups, the cysteinyl thiol and lysinyl ε-amino group (Guengerich and Liebler, 1985). Glutathione possesses both these nucleophilic groups, and so may be able to react with both hard and soft electrophiles. Glutathione is present at high concentrations in cells (e.g., about 10 mM in the liver) and its capacity to form amine-adducts may indicate a hitherto unrecognized capability to inactivate hard electrophiles such as certain aldehydes like the FPCs. This appears to be the first report of glutathione forming an adduct with an electrophilic xenobiotic via its amine group rather than the thiol.

The findings of this study indicate that the cellular target of antifeedant FPCs are amino groups on critical molecules, such as lysinyl ε-amino groups on enzymes. The facile binding of FPCs, and absence of their metabolites in excreta, suggest that the antifeedant effects occur in the gastrointestinal tract itself. Loss of enzyme function may lead to altered cellular metabolism and the release of chemical mediators such as 5HT, resulting in contraction of gastrointestinal smooth muscle and stimulation of sensory neurons. The animal may experience colic, nausea, and a general malaise, all contributing to the antifeedant effect of FPCs.

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INTERACTIVE EFFECTS OF GENOTYPE, ENVIRONMENT, AND ONTOGENY ON RESISTANCE OF CUCUMBER (*Cucumis sativus*) TO THE GENERALIST HERBIVORE, *Spodoptera exigua*

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Abstract—Host-plant genotype, environment, and ontogeny all play a role in determining plant resistance to herbivory, yet little is known about the nature of the interactions among these factors. We investigated resistance of cucumber plants *Cucumis sativus* to the generalist herbivore *Spodoptera exigua* in a manipulative experiment involving three factors. In particular, we tested the effects of bitter (cucurbitacins present) vs. sweet (cucurbitacins absent) plants (genotype), with or without previous herbivory (environment), and cotyledons vs. true leaves (ontogeny). Contrary to our expectations, *S. exigua* growth was 54% higher on bitter plants than on sweet plants; growth was 63% higher, however, on undamaged plants compared to damaged plants, and 59% higher on true leaves compared to cotyledons. Moreover, all two-way interaction terms between genotype, environment, and ontogeny were significant. For example, *S. exigua* performance was higher on bitter than on sweet plants; however, this effect was strongly influenced by whether the tissue consumed was a cotyledon or true leaf and also whether it had been previously damaged. An examination of leaf nutritional chemistry revealed that some of our results could be explained by genotypic, environmental, and ontogenic differences in foliar nitrogen content. In contrast, the cucurbitacin content of plants did not appear to affect caterpillar growth. Our results provide evidence for the importance of interactions between genotype, environment, and ontogeny in determining herbivory and illustrate the value of manipulative experiments in revealing the complexities of these interactions.

Key Words—Cucurbitacins, genotype by environment interaction, herbivory, nitrogen concentration, plant–insect interactions.

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INTRODUCTION

Determining the mechanisms of plant resistance to insects is a central pursuit in understanding the ecology and evolution of plant–herbivore interactions. Host-plant genotype, environment, and ontogeny are all known to play a role in influencing plant traits such as defense against herbivores (Denno and Mcclure, 1983; Fritz and Simms, 1992; Schlichting and Pigliucci, 1998). Genetically based variation in host-plant chemistry can result in large differences in plant resistance to herbivores (Karban, 1992). In addition, both the biotic and abiotic environment are well known to influence the defensive phenotype of plants (Karban and Baldwin, 1997; Koricheva et al., 1998a,b). Less is known about the influence of plant ontogeny or developmental stage on resistance to herbivory. All individuals pass through a series of ontogenic stages during growth, and recent studies indicate that differences in the developmental phase of plants can strongly influence resistance to herbivory (Kearsley and Whitham, 1989, 1998; Karban and Thaler, 1999; Gianoli, 2002). Although each of these factors has been examined individually, how they interact to determine levels of plant resistance is rarely studied. Understanding the interactions is critical for developing a predictive framework for the influence of plant traits on herbivores in the field, where plants show considerable variation in genotype, environment, and ontogeny.

Here, we use the cultivated cucumber *Cucumis sativus* (Cucurbitaceae) and the generalist herbivore *Spodoptera exigua* (Noctuidae) to determine how plant genotype, environment, and ontogeny interact to influence resistance to herbivory. *Spodoptera exigua* is a generalist herbivore known to feed on plants of at least 20 families, including the Cucurbitaceae (Tietz, 1972). Cucumber varieties vary qualitatively in the presence or absence of defensive cucurbitacins (tetracyclic triterpenoids), as determined by a single diallelic locus (Andeweg and De Bruyn, 1959). Cucurbitacins are bitter tasting, and plants that produce these compounds (hereafter bitter plants) are typically hostile hosts to a wide range of herbivores, including beetles, lepidopteran larvae, cockroaches, mice, and vertebrate grazers (Da Costa and Jones, 1971; Gould, 1978; Metcalf and Lampman, 1989; Tallamy and Krischik, 1989; Tallamy et al., 1997; Agrawal et al., 1999). In contrast, plants that produce no cucurbitacins (hereafter sweet plants) are typically more susceptible to herbivory (but see Chambliss and Jones, 1966; Metcalf et al., 1980; Agrawal et al., 1999). The occurrence of a simply inherited chemical polymorphism provided us with an experimental system for investigating how genotypes that differ in chemical resistance to herbivory are affected by environmental and ontogenic factors.

Several environmental influences have the potential to modify the intensity of herbivory on bitter vs. sweet plants of *C. sativus*. For example, herbivory typically induces responses in plants that negatively impact the preference and performance of subsequently feeding herbivores (Karban and Baldwin, 1997; Agrawal, 1998,

1999). In a comparison of bitter vs. sweet genotypes of *C. sativus*, Agrawal et al. (1999) demonstrated that spider mite herbivory decreased following damage, but this effect was only expressed in bitter plants. To what extent this type of interaction can be modified during plant ontogeny has not been investigated. However, it seems likely that since the chemical composition and hence food quality of plants change during development, these interactions with herbivores should be influenced by ontogeny. Indeed, Agrawal et al. (1999) found that cucurbitacin content varied greatly according to ontogenic stage, with cotyledons containing well over 10 times the cucurbitacins in true leaves. Also, when cotyledons were damaged, cucurbitacins increased by 30%, compared to a 50% increase in true leaves (Agrawal et al., 1999). These cucurbitacins have recently been shown to quantitatively reduce spider mite survival (Balkema-Boomstra et al., 2003).

In this study, we asked the following questions: (1) Is the performance of *S. exigua* reduced on bitter vs. sweet genotypes? (2) Does previous herbivory by *S. exigua* cause an induced response that reduces the performance of subsequent herbivores of this species? (3) Does *S. exigua* exhibit a preference for feeding on different ontogenic stages (cotyledons vs. true leaves) of *C. sativus*, and how does this influence their performance? We then considered pair-wise interactions between each of these factors to evaluate whether environmental and ontogenic factors influencing food quality might alter the performance of *S. exigua* on bitter vs. sweet plants. Finally, we conducted chemical analyses to determine the carbon and nitrogen concentrations of leaves as influenced by genotype, environment, ontogeny, and their interactions. Examination of these primary nutrients, which can be limiting for herbivores, complements our previous analyses of how genotype, environment, and ontogeny influence defensive cucurbitacins (Agrawal et al., 1999).

METHODS AND MATERIALS

Study System and General Procedures. We conducted all experiments using the near-isogenic varieties Marketmore 76 (bitter) and Marketmore 80 (sweet) of the cultivated cucumber (*C. sativus*) (Gould, 1978; Agrawal et al., 1999, 2002). A single diallelic locus controls the presence or absence of cucurbitacin production in this species, with the dominant allele governing synthesis of cucurbitacins (Andeweg and De Bruyn, 1959). All experiments were conducted during June–August 2002 at the Koffler Scientific Reserve at Jokers Hill, near Newmarket, Ontario, Canada (44°03′ N, 79°29′ W). Cucumber plants were grown from seed, germinated on petri dishes, and grown in 210-ml pots using a Pro-Mix soil (Red Hill, PA) and ≈0.3 g of slow-release Nutricote fertilizer (13:13:13::N:P:K) (Vicksburg Chemical, Vicksburg, MS). We typically grew plants in a completely randomized design under field conditions in large enclosures ($2 \times 2 \times 3$ m in size) made of nylon mesh to exclude wild herbivores.

We maintained *S. exigua* in a laboratory colony on an artificial diet (Southland Products, Arkansas). Third to fifth instar larvae were used to damage plants (for induced resistance experiments) and freshly hatched neonates were used for all bioassays of plant resistance. In each bioassay, we typically allowed a single larva to feed for 5 days and used the fresh mass of the caterpillars as our response variable. We conducted bioassays on cut leaves placed in 90-mm Petri dishes lined with moistened filter paper and sealed with Parafilm. In such bioassays, we used a single replicate of cotyledons and true leaves from each plant.

Effects of Genotype, Environment, and Ontogeny on S. exigua. To determine the independent and interactive effects of genotype, environment, and ontogeny on *S. exigua* performance, we conducted two experiments. In the first, we tested whether genotype and ontogeny influenced *S. exigua* performance on *C. sativus* plants grown within enclosures. We used a total of 80 plants distributed equally among the four treatment combinations (bitter vs. sweet and cotyledon vs. true leaf). We measured *S. exigua* growth and mortality on cotyledons or true leaves from bitter and sweet plants using the bioassay procedure described earlier. We assessed differences in mortality using a *G* test and growth using two-way ANOVA.

In the second experiment, we tested for the effects of all three factors (bitter vs. sweet, damaged vs. undamaged, and cotyledon vs. true leaf) and their interactions on *S. exigua* using the protocols described earlier. To condition the damaging caterpillars to cucumber, we placed them in a container with detached cucumber leaves for 24 hr before use. The caterpillars were allowed to damage singly bagged plants for 1 wk and were removed when approximately 10% of total leaf area (estimated visually) was removed per plant. We conducted bioassays 2 days following removal of the caterpillars. We used a total of 160 plants in this experiment, with sample size balanced among the eight treatment combinations. We analyzed data from this experiment using a three-factor ANOVA including all interactions.

To determine if *S. exigua* exhibited a preference for cotyledons or true leaves, we compared the total amount of herbivory on these two developmental stages by third to fifth instar *S. exigua* in the experiment described earlier. In addition, we conducted a separate experiment with a total of 58 *C. sativus* plants that were transplanted into a ploughed field at the cotyledon stage and grown with 1-m spacing between plants. We covered all plants with spun polyester sleeves (Rockingham Opportunities Corporation, Reidsville, NC) to protect them from herbivory. Single third to fifth instar *S. exigua* were placed on plants with at least two true leaves, and caterpillars were replaced if they escaped. For each replicate, preference for cotyledons vs. true leaves was determined by visual estimation of the plant part that had greater leaf area removed. We assessed preference using a *G* test with William's correction; replicates with no herbivory were eliminated from the analysis.

Effect of Genotype on Generalist Weevil Preference and Spider Mite Performance. Because of our nonintuitive results of *S. exigua* having higher performance on bitter plants (see results), we tested how two other generalist herbivores were

influenced by the presence of cucurbitacins. In particular, we investigated whether the feeding preference of weevils or the fecundity of spider mites differed between bitter and sweet plants of *C. sativus*. We used a naturally occurring weevil (*Sciaphilus asperatus*) from our study site and spider mites (*Tetranychus urticae*) obtained from a laboratory colony maintained on cultivated cotton plants. Both of these herbivores have broad host ranges and feed on plants in many families. In the feeding preference experiment, we recorded whether weevils fed on bitter ($N = 26$) or sweet ($N = 16$) plants grown within a single enclosure ($2 \times 2 \times 3$ m). Plants with two to three true leaves were randomized within the enclosure, and greater than 20 weevils were foraging inside. After 2 wk, we recorded whether weevils had imposed visible damage on each plant. We determined the effects of genotype on herbivore preference (damaged vs. undamaged) using a G test.

For the separate experiment using spider mites, we conducted two bioassay trials to test if plant genotype had an effect on mite fecundity. Previous experiments have established strong negative effects of cucurbitacins on spider mites (Agrawal et al., 1999). We inoculated cotyledons of nearly equal numbers of bitter and sweet plants with three adult female spider mites ($N = 29$ for trial 1 and $N = 47$ for trial 2). We counted the number of eggs on each plant after 1 wk and tested for the effect of plant genotype and trial on mite fecundity using a factorial ANOVA.

Plant Chemistry: Analysis of Carbon and Nitrogen Content of Leaves. To determine whether the nutritional status of leaves may be influenced by plant genotype, environment, and ontogeny, we examined the carbon and nitrogen concentrations of leaves. In particular, we were interested in whether the positive nutritional impact of nitrogen modified herbivore performance and thereby counteracted the influence of other factors such as cucurbitacins. We investigated the effects of all three factors and their interactions on leaf chemistry by growing and treating 60 plants as above, equally distributed among the eight treatment combinations (bitter vs. sweet, damaged vs. undamaged, and cotyledon vs. first true leaf). We separated true leaves and cotyledons and stored the samples in a -20°C freezer. Total foliar carbon and nitrogen concentrations were analyzed using a Perkin-Elmer CHNS Elemental analyzer with autosampler at the Analest facility, University of Toronto. We analyzed data from this experiment using a three-factor ANOVA including all interactions.

RESULTS

Plant Genotype and Performance of S. exigua. In contrast to our prediction, performance of *S. exigua* on bitter plants was generally higher than on sweet plants. The mortality of neonates was one-third lower ($G = 21.81$, $P < 0.001$), and the growth of survivors was 54% higher (separate variance $t = 2.52$, $df = 41.9$, $P = 0.016$) when feeding on bitter compared to sweet plants (Figure 1). In this first experiment, ontogeny had no effect on *S. exigua* performance ($P > 0.100$).

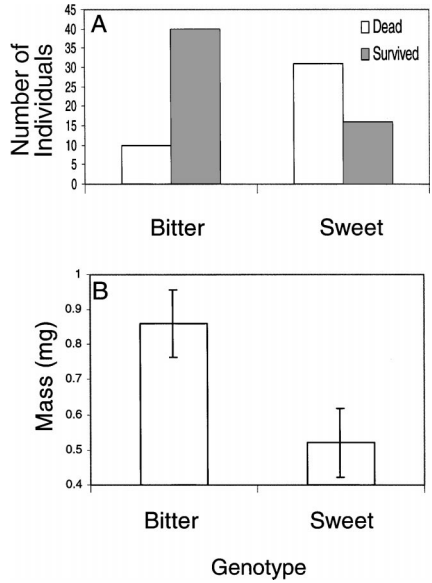

FIG. 1. The influence of plant genotype (leaves from bitter or sweet plants) on (A) mortality and (B) mass of surviving *Spodoptera exigua* (mean ± SE).

Interactive Effects of Genotype, Environment, and Ontogeny on S. exigua *Performance.* Genotype, environment, and ontogeny all influenced *S. exigua* performance in the experiment involving all three factors (Table 1, Figures 2 and 3). In common with the preceding experiment, *S. exigua* grew 16% faster on bitter than on sweet plants (Table 1, Figure 2). As predicted, growth was 63% higher on undamaged plants compared to damaged plants, providing evidence for induced resistance to herbivory (Table 1, Figures 2 and 3). However, in contrast to the

TABLE 1. ANALYSIS OF VARIANCE FOR THE EFFECTS OF GENOTYPE (BITTER OR SWEET), ENVIRONMENT (INDUCED OR CONTROL) AND ONTOGENY (COTYLEDON OR TRUE LEAF) ON *Spodoptera exigua* GROWTH

Source	SS	*df*	MS	*F*	*P*
Plant genotype (G)	0.407	1	0.407	5.860	0.016
Herbivore induction (I)	1.978	1	1.978	28.479	<0.001
Ontogeny (O)	11.285	1	11.285	162.510	<0.001
G × I	0.258	1	0.258	3.720	0.055
G × O	0.498	1	0.498	7.178	0.008
I × O	0.359	1	0.359	5.165	0.024
G × I × O	0.063	1	0.063	0.900	0.343
Error	19.860	286	0.069		

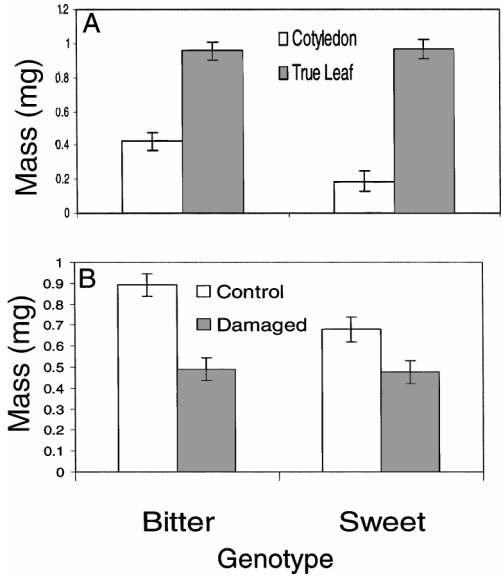

FIG. 2. The influence of plant genotype (leaves from bitter or sweet plants), ontogenic position (cotyledon or true leaf), and induction treatment (with or without previous damage by *Spodoptera exigua*) on mass of neonate *S. exigua* (least-squares means ± SE). Significant interactions are depicted in the figures: (A) the effect of genotype and ontogenic position on *S. exigua* growth; (B) the effect of leaf genotype and induction treatment on *S. exigua* growth.

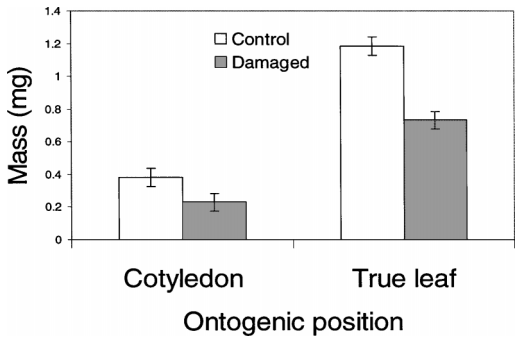

FIG. 3. The interactive effect of ontogenic position (cotyledon or true leaf) and induction treatment (with or without previous damaging by *Spodoptera exigua*) on growth of neonate *S. exigua* (least squares means ± SE).

preceding experiment, we detected a significant difference in performance of *S. exigua* neonates on the two developmental stages of *C. sativus* (Table 1). Caterpillar growth on true leaves was 59% higher than on cotyledons. It is unclear what caused this differential result between the two trials.

All three of the two-way interactions were significant (Table 1) indicating complex relations among genotype, environment, and ontogeny. For example, we found that *S. exigua* performance was higher on bitter vs. sweet plants; however, this effect was strongly influenced by whether the tissue consumed was a cotyledon or true leaf, and also whether or not it had been previously damaged (Figure 2). In particular, the increased performance seen on bitter plants was only apparent on cotyledons and undamaged plants. Similarly, ontogenic stage significantly affected *S. exigua* growth, but this response differed between previously damaged vs. undamaged plants (Figure 3). The increased performance on true leaves compared to cotyledons was much greater on control plants than on previously damaged plants.

Spodoptera exigua herbivory on bitter plants grown in the field was 10 times greater on cotyledons than on true leaves (Figure 4, adjusted $G = 58.253$, $P < 0.001$). A similar pattern of herbivory was evident on bitter plants in the enclosure (adjusted $G = 7.819$, $P = 0.005$) but not on sweet plants (Figure 4, adjusted $G = 0.116$, $P = 0.733$).

Plant Genotype and Weevils and Spider Mites. Results from two other generalist herbivores feeding on *C. sativus* differed from those described for *S. exigua*. Weevils from a natural population at our study site showed a clear preference for sweet plants (70% damaged) over bitter plants (11% damaged) ($G = 14.996$, $P < 0.001$). Spider mites also performed better on sweet plants than bitter plants, with higher fecundity during the 1-wk observation period (mean $\pm$ SE eggs, Bitter: 58.745 ± 3.741, Sweet: 71.546 ± 3.632; Genotype $F(1, 72) = 6.027$, $P = 0.017$;

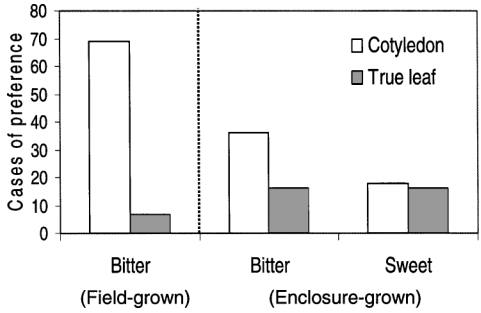

FIG. 4. Preference of *Spodoptera exigua* on bitter and sweet plants for feeding on cotyledons vs. true leaves.

TABLE 2. ANALYSIS OF VARIANCE FOR THE EFFECTS OF GENOTYPE (BITTER OR SWEET), ENVIRONMENT (INDUCED OR CONTROL) AND ONTOGENY (COTYLEDON OR TRUE LEAF) ON NITROGEN CONTENT IN *Cucumis sativus*

Source	SS	*df*	MS	*F*	*P*
Plant genotype (G)	5.396	1	5.396	14.145	<0.001
Herbivore induction (I)	2.653	1	2.653	6.955	<0.001
Ontogeny (O)	21.357	1	21.357	55.985	<0.001
G × I	1.475	1	1.475	3.867	0.052
G × O	0.200	1	0.200	0.524	0.471
I × O	0.297	1	0.297	0.779	0.380
G × I × O	0.035	1	0.035	0.092	0.762
Error	33.570	1	0.381		

Trial $F(1, 72) = 63.903$, $P < 0.001$; Genotype × Trial, $F(1, 72) = 0.226$, $P = 0.636$).

Plant Chemistry: Analysis of Carbon and Nitrogen Content of Leaves. Genotype, environment, and ontogeny all significantly influenced nitrogen concentration (Table 2, Figure 5), but only ontogeny had any effect on carbon concentration (least-squares means ± SE percentage carbon, cotyledons: 38.9 ± 0.3, true leaves: 43.9 ± 0.3, $F(1, 88) = 8.987$, $P < 0.001$, all other P values in the fully factorial analysis >0.100). Bitter plants showed 12% higher nitrogen concentration than the sweet genotype. Nitrogen concentration was 8% higher on damaged compared to undamaged plants. In addition, true leaves contained 22% higher nitrogen concentration and 11% higher carbon concentration than cotyledons (Figure 6). Finally,

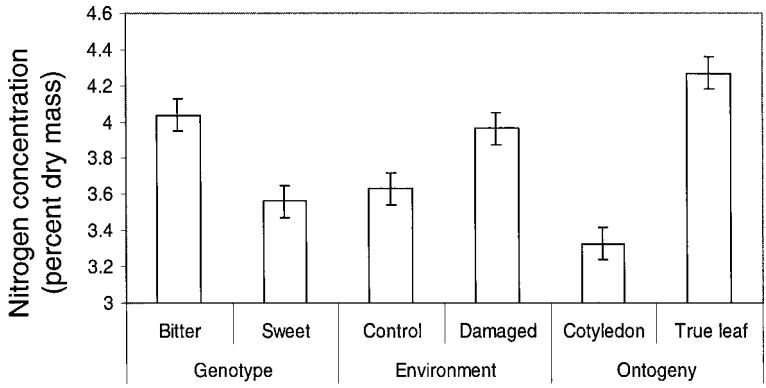

FIG. 5. Nitrogen concentration of leaves as affected by plant genotype (bitter or sweet), ontogenic position (cotyledon or true leaf), and induction treatment (with or without previous damaging by *Spodoptera exigua*). Overall main effects are reported in the figure (averaging over other factors, as calculated by least squares means ± SE).

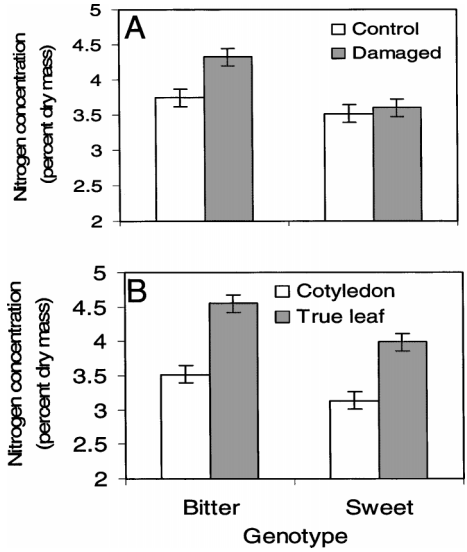

FIG. 6. The effect of cucumber genotype (bitter or sweet) and (A) damage state or (B) ontogenic position (cotyledon or true leaf) on nitrogen content (least squares means ± SE).

there was a significant interaction between the effects of genotype and previous damage on nitrogen concentration (Figure 6). Damaged bitter plants showed 17% higher nitrogen concentration than damaged sweet plants; however, this difference was minimal (6%) in undamaged plants.

DISCUSSION

The role of genotype, environment, and ontogeny in determining plant and animal phenotypes is widely recognized (Schlichting and Pigliucci, 1998). Yet, experimental investigations of herbivory rarely capture this ecological realism. The results of our study implicate complex interactions among all three factors on generalist herbivore feeding. This finding has important implications for understanding the variation in intensity of herbivory that is commonly observed in the field. Later we discuss how factors influencing host-plant chemistry may have played a decisive role in the complexity of our findings.

Why Does S. exigua *Perform Better on Bitter vs. Sweet Plants?* Contrary to our predictions, *S. exigua* exhibited higher performance on the bitter than on the sweet genotype of *C. sativus*. In experiments with adult *S. exigua*, Tallamy et al. (1997) found that cucurbitacins deterred oviposition. We expected that larval *S. exigua*, like other generalist herbivores, would also be deterred by the presence of cucurbitacins in bitter plants. Indeed, both of the other generalist herbivores

(weevils and spider mites) either preferred or performed better on sweet compared to bitter plants. This suggests that the response of *S. exigua* larvae on *C. sativus* may be atypical for a generalist (Tallamy et al., 1997). Insights into the potential explanation for this unexpected response can be obtained by examining the interactions between genotype and the other two factors investigated.

The benefit that *S. exigua* gained on bitter plants was only evident when larvae were feeding on either cotyledons or undamaged plants. In contrast, the difference in performance on the two genotypes was significantly less when larvae fed on true leaves or on damaged plants. This difference in when bitterness affected *S. exigua* was associated with contrasting nitrogen levels in plants. Our chemical analysis (Figures 5 and 6) indicated that cotyledons and undamaged plants possessed lower levels of nitrogen than true leaves and damaged plants, respectively. Moreover, we also found that overall bitter plants had significantly higher nitrogen content than sweet plants. Therefore, since nitrogen is almost certainly a limiting nutrient for *S. exigua* (Al-Zubaidi and Capinera, 1983; Broadway and Duffey, 1988), it is plausible that larvae performed better on bitter plants because of the additional nitrogen. In addition, our bioassays were conducted with newly hatched first instars, which may be particularly sensitive to low nitrogen concentrations in leaves (Zalucki et al., 2002).

Spodoptera exigua only performed better on bitter plants in treatments in which the bitter genotype was paired with low nitrogen states (undamaged plants and cotyledons). This finding suggests that in these combinations, higher nitrogen in bitter plants was important as a source for enhanced performance of the caterpillars (Figure 6). Conversely, there was no enhancement of performance on bitter vs. sweet plants where larvae fed on damaged bitter plants or their true leaves, presumably because nitrogen demand was met by factors other than genotype. These contrasting responses suggest that the detrimental effects of cucurbitacins in the bitter genotype can be countered by increased performance, resulting from high nitrogen, but only when other factors limit this essential nutrient. This explanation implies that the effectiveness of chemical defenses may be modified depending on the nitrogen composition of the plants employing them (Broadway and Duffey, 1988).

The underlying causes for nitrogen differences among the treatments are unknown. It may be argued that since nitrogen concentrations are higher in bitter plants and in induced bitter plants (but not in sweet plants, Figure 6), the cucurbitacins are responsible for elevating nitrogen concentrations. However, cucurbitacins may have a pleiotropic effect influencing other traits affecting nitrogen production. Alternatively, the genes that are tightly linked to the gene that controls the production of cucurbitacins may influence other traits affecting nitrogen production.

A potential explanation for the higher nitrogen concentrations present in damaged plants could be that previously damaged plants induced nitrogen-rich

compounds in addition to the carbon-based cucurbitacins. Although herbivory has been reported to both increase and decrease nitrogen concentrations, depending on the plant–herbivore system (Murray et al., 1996; Ruohomaki et al., 1996; Danell et al., 1997; Karban and Baldwin, 1997), cucumbers in particular increase photosynthetic efficiency following herbivory (Thomson et al., 2003). Such increases in photosynthetic efficiency are frequently due to increases in foliar Rubisco levels, which are rich in nitrogen (Lambers et al., 1998). The effect of ontogeny on nitrogen concentration is more difficult to explain. Karban and Thaler (1999) showed that cotton cotyledons have an increased rate of photosynthesis compared to true leaves, and suggested that this may be responsible for the higher level of herbivory that was observed. It is possible that some process or attribute correlated with photosynthesis (i.e., chlorophyll concentrations) is accountable for altering the nitrogen concentrations of different plants or plant parts.

Induced Responses and Ontogeny Modify Herbivore Performance. As expected, the larvae of *S. exigua* feeding on undamaged plants performed better than those on previously damaged plants. This finding is consistent with induced defense theory, which predicts that following an attack, defenses are mounted that serve to increase the resistance of plants to further herbivory (Karban and Baldwin, 1997; Agrawal, 1998). In our case, we assume that the induced compounds involved elevated cucurbitacins, as was demonstrated in an earlier study with the same varieties (Agrawal et al., 1999). Since only bitter plants of *C. sativus* produce cucurbitacin, we expected no difference in the performance of *S. exigua* on damaged vs. undamaged plants of the sweet genotype. However, the presence of a small decrease in *S. exigua* performance on damaged sweet plants in relation to undamaged plants suggests that there may be other detrimental induced responses in *C. sativus*. Indeed, in an analysis of the head-space volatiles produced by damaged bitter and sweet plants, we found several induced compounds in sweet plants (Agrawal et al., 2002). The greater strength of induced resistance in bitter plants than sweet plants presumably results from an interaction of several factors including changes in cucurbitacins, nitrogen, and other unknown factors.

Our results indicate that induced response may also be modified by plant ontogeny. *Spodoptera exigua* performance on undamaged plants was significantly higher than damaged plants when larvae fed on true leaves. In contrast, there was little difference in larval performance when larvae fed on cotyledons, irrespective of whether plants were damaged or not (Figure 3). This result may be associated with the interaction between nitrogen content, ontogeny, and the mechanisms of induced resistance. In a study on plant phase change and resistance to herbivory, Karban and Thaler (1999) demonstrated that population growth of spider mites on cotton is greater on cotyledons than on true leaves, and that this difference is likely due to some process correlated with high photosynthetic rates in cotyledons. Cucurbitacin content also varies with ontogenic stage (Agrawal et al., 1999). The extent to which *S. exigua* performed better on undamaged plants appears to be moderated

by whether damaged or undamaged plants are combined with a high (true leaves) or low (cotyledons) nitrogen factor. We speculate that since undamaged plants have lower nitrogen content than damaged plants, the positive advantage these plants confer to *S. exigua* from the absence of induced defenses is offset by their lack of nitrogen. This may explain why, if nitrogen deficiency is removed through combination with a high nitrogen factor (i.e., true leaves or the bitter genotype), the undamaged plants confer an increased advantage over damaged plants.

Our results suggest that plants in the field are likely to provide diverse food choices for the same species of generalist herbivore depending on their developmental status and prior history of herbivory. Interestingly, in preference tests, *S. exigua* overwhelmingly chose to feed on cotyledons over true leaves, even though their performance is lower on this ontogenic stage. Although this is counterintuitive, previous studies have also found that individual preference and performance are not always linked in herbivory (Waldbauer and Friedman, 1991; Karban and Agrawal, 2002). For example, *Smynthurodes betae* galling aphids did not display any preference for optimal galling sites on leaves (Burstein and Wool, 1993).

Interactions Between Genotype, Environment, and Ontogeny Maintain Variation in Food Quality. Our results have highlighted the complex interactions that can occur among genotype, environment, and ontogeny in governing generalist herbivore performance. There was no single combination of the three factors in our experiments that optimized larval feeding on *Cucumis sativus*. This implies that in natural populations where there is considerable environmental heterogeneity and variation in developmental status of plants, herbivores are likely confronted with a large range of food choices resulting from the changing nutritional profiles of plant material. This pervasive spatial and temporal variation within and between plants may make it quite difficult for herbivores to optimally choose their foods and may ultimately be a resistance mechanism itself (Denno and Mcclure, 1983; Whitham, 1983; Karban and Baldwin, 1997; Agrawal and Karban, 1999; Orians and Jones, 2001; Orians et al., 2002).

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ABOVE- AND BELOW-GROUND TERPENOID ALDEHYDE INDUCTION IN COTTON, *Gossypium herbaceum*, FOLLOWING ROOT AND LEAF INJURY

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Abstract—Studies on induced defenses have predominantly focused on foliar induction by above-ground herbivores and pathogens. However, roots are attacked by as many if not more phytophages than shoots, so in reality plants are exposed to above- and below-ground attack. Here, we report effects of foliar and/or root damage on terpenoid aldehyde accumulation in cotton (*Gossypium herbaceum*). Using HPLC, we analyzed concentrations of individual terpenoid aldehydes in foliage and root tissue. In undamaged plants, terpenoid aldehydes were concentrated in young immature main leaves. Concentrations in side leaves, branching from the main leaves, did not differ among leaf position. Above-ground feeding by *Spodoptera exigua* larvae on a mature leaf enhanced terpenoid concentrations in immature leaves but not in the damaged leaf. In particular, concentrations of hemigossypolone and the heliocides 1 and 4 were enhanced following herbivory. Root herbivory by wireworms (*Agriotes lineatus*) also resulted in an increase in terpenoid levels in the foliage. In contrast with foliar herbivory, both immature and mature leaves were induced. However, the level of induction after root herbivory was much lower compared to foliar herbivory. Plants exposed to root herbivory also had significantly higher levels of terpenoid aldehydes in root tissue, while no such effect was found following foliar herbivory. Plants exposed to both root and foliar herbivory appeared to

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induce primarily above-ground at the cost of below-ground defense. The implications for above- and below-ground Mutitrophic interactions are discussed.

Key Words—*Agriotes lineatus*, allocation, gossypol, heliocide, hemigossypolone, induced defense, mechanical damage, spatial distribution, *Spodoptera exigua*, systemic induction.

INTRODUCTION

Insect herbivory can induce different chemical defense responses in root and shoot tissue (Karban and Baldwin, 1997; van Dam et al., 2003). Roots are attacked by at least as many herbivores as are shoots (Brown and Gange, 1990; van der Putten et al., 2001). Most studies, however, have focused on induced chemical defenses in shoots and leaves by above-ground herbivores and pathogens (Bezemer et al., 2003; van Dam et al., 2003). A distinction is often made between local and systemic induction (Edwards and Wratten, 1983). Systemic induction can be uniform throughout the plant or vary between different plant parts (Jones et al., 1993; Stout et al., 1996). The optimal defense theory (e.g., Zangerl and Rutledge, 1996; Ohnmeis and Baldwin, 2000) suggests that the greatest accumulations of secondary defense compounds often occur in the most valuable parts of a plant such as seeds, flowers, and young leaves (Zangerl and Bazzaz, 1992; Hartley and Jones, 1997).

Cotton (*Gossypium* spp.) produces terpenoids, e.g., desoxyhemigossypol, hemigossypol, and gossypol in the roots, and hemigossypolone, the heliocides H_1, H_2, H_3, and H_4 and in lower concentration gossypol in foliage (Stipanovic et al., 1988; Liu et al., 1999) that defend the plant against a wide range of herbivores and pathogens (Stipanovic et al., 1977, 1978a,b; Bell et al., 1978; Khan et al., 1999). Cotton plants store terpenoid aldehydes in pigmented glands in various above-ground plant structures, and lepidopteran larvae, including tobacco budworm, *Heliothis virescens* (F.), avoid eating pigment glands (Hedin et al., 1992). When terpenoids are incorporated into artificial diet, larvae perform poorly, showing increased developmental time, increased mortality, and reduced larval and pupal weights (Stipanovic et al., 1977).

Individual terpenoid aldehydes differ in their effects on herbivores and pathogens (Stipanovic et al., 1990; Hedin et al., 1992; Zhang et al., 1993). The precursors desoxyhemigossypol and hemigossypol that occur in cotton roots, for example, are highly toxic to seedling pathogens, while the end product, gossypol, inhibits root pathogens only at a greater concentration (Stipanovic et al., 1990). In foliage, the heliocides and gossypol retard tobacco budworm growth equally, but hemigossypolone, the precursor of the heliocides, is less effective (Stipanovic et al., 1990). Feeding assays have also revealed that heliocides 1 and 4 cause significantly higher reduction in growth of larval *Heliothis zea* (Boddy) than heliocides 2 and 3 (Stipanovic et al., 1977, 1978a,b).

The purpose of this study was to investigate induction and spatial distribution of terpenoids, resulting from different types of damage to cotton plants using above- and below-ground herbivorous arthropods.

METHODS AND MATERIALS

Plants. Cotton, *G. herbaceum*, plants were grown in a greenhouse at 60% RH, 16L:8D, and 23°C (±1°C) during the day and 18°C (±1°C) at night. The greenhouse was equipped with 400 W metal halide bulbs (1/1.5 m^2). To avoid root limitation, plants were grown individually in 7-l pots in a 50:40:10 peat:sand:clay mixture. Plants were not supplied with fertilizer and were watered once every 2 days.

Herbivores. Wireworm, *Agriotes lineatus* (L.) (Coleoptera: Elateridae) larvae were collected by hand-sorting soil samples taken from a pasture in Wageningen, The Netherlands, with a density of about 20 larvae per m^2. Late-instar individuals were starved for 3 days in moist soil and weighed using a microbalance before introduction into the pots in which the plants were grown. Seven individuals were introduced in each pot by placing them on the soil surface, whereafter they burrowed into the soil. Beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) larvae, were reared from hatching on excised cotton leaves. Third instars were used in all experiments. Individual larvae were placed in clip-on cages (2.5 cm^2 diam.; Bezemer et al., 1999) to standardize the level of above-ground herbivory. The connecting inner sides of the cages were coated with foam rubber to prevent additional leaf damage. The outsides were covered with screen mesh.

Above-ground Distribution of Terpenoids After Foliar Herbivory. All plants had eight true leaves at the start of the assay. The third true leaf of five plants was exposed to foliar herbivory by using four clip-on cages per plant. Within each clip-on cage there was a beet armyworm larva. Five other plants were not damaged. After 2 days, the leaf area inside each clip-on cage was consumed, and the cages were removed. Seven days later, all leaves (two cotyledons, all main leaves, and, if present, side leaves appearing from the growth point of each individual main leaf or cotyledon) were excised from the stem with a razor blade. The leaves were freeze-dried, ground, and extracted individually for terpenoid analysis. Terpenoid aldehyde levels of each leaf were analyzed using Analysis of Variance (ANOVA) with leaf position on the plant and herbivory (present or absent) as main factors. If there was a significant herbivory effect or interaction, for each position on the plant, the effect of herbivory on terpenoid concentrations was also tested by using a *t*-test. Main leaves (including cotyledons) and side leaves were analyzed separately.

Combined and Isolated Effects of Root and Shoot Herbivory on Terpenoid Accumulation. Forty 15-day-old plants were exposed for 5 wk to root herbivory by wireworms, foliar herbivory by beet armyworm larvae, or both or neither ($n = 10$). At the start of the experiment, plants had two real leaves, and at the end of the

experiment there were on average 12 real leaves. For plants exposed to beet armyworm herbivory, a clip-on cage with one beet armyworm larva inside was placed on the third mature leaf for a period of 2 days. When the total leaf area within the cage was consumed, the larva was placed on a new area of the same leaf. The larva was moved in the clip-on cage to the next mature leaf after 5 cm^2 of leaf area (two feeding holes) was consumed. A total of 20 cm^2 was consumed on four leaves per plant. Five weeks after the herbivore treatments began, the one but youngest mature leaf, and damaged, and the oldest immature leaf, nondamaged, were excised from each plant using a razor blade. Samples were immediately frozen at $-80^{\circ}C$. The samples were later freeze-dried, ground, and extracted for terpenoid analysis.

Roots of all plants were cut from the stem at the soil surface, washed, and separated into main and lateral roots. All lateral root material was freeze-dried, ground, and analyzed for terpenoids. The effects of root and shoot herbivory on levels of individual terpenoid aldehydes detected in root, mature leaf, and immature leaf tissue were first analyzed using a two-way MANOVA (Wilks' test). Differences in individual terpenoid aldehydes were analyzed separately using ANOVA. To ascertain differences in terpenoids between mature and immature leaves, these data were also analyzed by using a three-way MANOVA with leaf (mature/immature), root, and foliar herbivory as main factors. Ratios between heliocides 1 and 4, and heliocides 2 and 3, and hemigossypolone and the total heliocides were compared by using ANOVA, and individual comparisons were made by using Tukey's HSD.

Effects of Mechanical Root Damage on Terpenoid Accumulation. A metal blade was used to cut a 12-cm-diam. circle around the main stem of 6-wk-old plants with eight true leaves ($n = 7$). As the diameter of the pot was 27 cm, all roots growing into the outer rim of 7.5 cm were cut. Other plants were exposed to beet armyworm larvae as described in the aboveground herbivory assay, or grown without mechanical root injury or herbivory. Nine days after injury commenced, the third mature leaf (damaged in plants exposed to foliar herbivory) and seventh immature leaf were excised, freeze-dried, and ground. The roots of all plants were washed, separated into main and lateral roots, and all lateral root material was freeze-dried, ground, and extracted for terpenoid analysis. The effects of root and shoot damage on levels of individual terpenoid aldehydes detected in root, mature leaf, and immature leaf tissue were first analyzed by using one-way MANOVA. Individual terpenoid aldehydes were also analyzed separately by using ANOVA. Individual comparisons were made by using Tukey's HSD.

Terpenoid Analysis. The high performance liquid chromatography (HPLC) procedure outlined by Stipanovic et al. (1988) was used to analyze terpenoid concentrations. Samples, each 100 mg of freeze-dried ground plant material, were shaken for 30 min in a capped 125-ml erlenmeyer flask with 15 ml of glass beads, 10 ml of 3:1 hexane: ethyl acetate (HEA) and 100 μl of 10% HCl. The solution was filtered over a glass-fritted filter funnel into a 50-ml pear-shaped flask, and the beads and residue were rinsed three times with 3-ml HEA. The solvent was left to

evaporate in a hot water bath, and the residue in the flask was redissolved with four 150-μl HEA washes. Each wash was transferred to a Maxi-clean silica cartridge (Alltech, Breda, The Netherlands). The silica cartridge was dried with compressed air, and terpenoid compounds were eluted with 5-ml isopropyl alcohol, acetonitrile, water, and ethyl acetate (35:21:39:5). The eluent was filtered through a 45-μm nylon filter and transferred to a crimp top vial for HPLC analysis. Twenty microliters of each sample were injected onto a DIONEX HPLC-system (DIONEX Corp., Sunnyvale, CA, USA), equipped with a single wavelength absorbance detector and a 250 mm $\times$ 4.6 mm i.d. Alltima C-18 column (Alltech, Breda, The Netherlands). The column was eluted with EtOH:MeOH:IPA:ACN:H_2O: EtOAc:DMF:PPAc = 16.7:4.6:12.1:20.2:37.4:3.8:5.1:0.1 at a flow rate of 1.25 ml min^{-1} and kept at 55°C during analysis (Stipanovic et al., 1988). Detection was at 272 nm. Standards of gossypol, hemigossypolone, hemigossypol, and heliocides 1 and 4, and heliocides 2 and 3 were used to assess retention times of the individual terpenoids. *G. herbaceum* roots also contain methoxyhemigossypol and mono- and dimethylgossypol-ether, but, since no standards were available for these compounds, the concentration of these compounds was calculated using the gossypol standard curve and hence expressed in gossypol equivalents (McAuslane et al., 1997).

RESULTS

Aboveground Distribution of Terpenoids After Foliar Herbivory. Levels of all detected terpenoid compounds in main leaves differed significantly between leaves: gossypol ($F_{8,62} = 13.11$, $P < 0.001$), hemigossypolone ($F_{8,62} = 46.05$, $P < 0.001$), heliocides 1 and 4 ($F_{8,62} = 12.29$, $P < 0.001$), and heliocides 2 and 3 ($F_{8,62} = 26.71$, $P < 0.001$) (Figure 1). Independent of whether plants had been exposed to herbivory or not, the highest accumulations of hemigossypolone, heliocides 1 and 4, and heliocides 2 and 3 were found in the youngest leaves. Accumulations of gossypol, however, were highest in cotyledons (Figure 1). Herbivory caused significant increases in hemigossypolone ($F_{1,62} = 23.47$, $P < 0.001$), heliocides 1 and 4 ($F_{1,62} = 24.45$, $P < 0.001$), and to a lesser extent heliocides 2 and 3 ($F_{1,62} = 5.66$, $P < 0.05$), while gossypol was not affected ($F_{1,62} = 0.21$, $P = 0.65$) (Figure 1). Heliocides 1 and 4 increased 3.4-fold and hemigossypolone increased 1.9-fold relative to control leaves. No effects of herbivory were found in the damaged leaf.

In side leaves, terpenoid accumulation in control plants was not different between leaf positions (Figure 1). Foliar herbivory caused significant increases in gossypol ($F_{1,48} = 8.27$, $P < 0.01$), hemigossypolone ($F_{1,48} = 35.01$, $P < 0.001$), and heliocides 1 and 4 ($F_{1,48} = 22.79$, $P < 0.001$) (Figure 1). This effect was most marked for heliocides 1 and 4 (2.8-fold increase) and hemigossypolone (1.8-fold

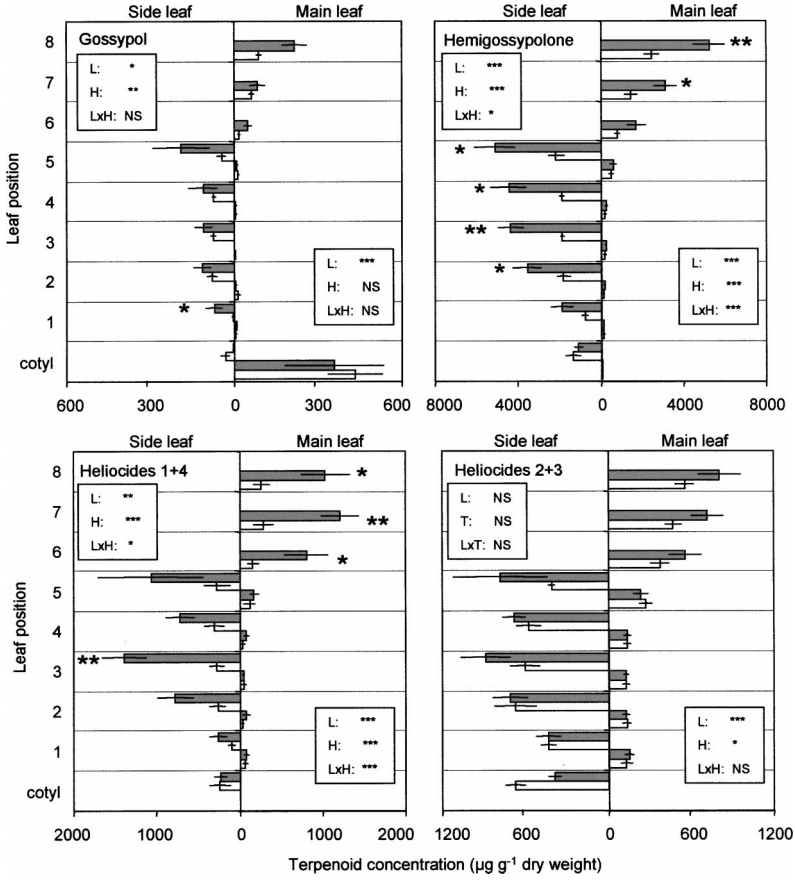

FIG. 1. Mean (±SE) concentration of terpenoid aldehydes in cotyledons (cotyl), main leaves, and side leaves appearing from the growth point of the cotyledon or main leaf of cotton plants. Plants were kept undamaged (□) or exposed to foliar herbivory for 48 hr on the third main leaf (■). Leaves are ranked from oldest (1) to youngest (8). Inserted are results of two-way ANOVA's testing the effect of leaf position (L); herbivory treatment (H). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns = no significant difference.

increase). For these compounds, the greatest effects of herbivory on terpenoid accumulation were in the side leaf at leaf position 3 with herbivore damage (Figure 1).

Combined and Isolated Effects of Root and Shoot Herbivory on Terpenoid Accumulation. Both types of herbivory resulted in increased terpenoid levels in foliage. The increase was highest for heliocides 1 and 4 (mature leaves 4.0 times higher than control, immature leaves 41.9 times higher than control). Terpenoid accumulations also differed between herbivory types (Table 1). In mature leaves,

TABLE 1. RESULTS OF MANOVA AND ANOVA TESTS OF THE EFFECTS OF ROOT AND FOLIAR HERBIVORY ON TERPENOID ACCUMULATION

		Root herbivory			Foliar herbivory			Root × foliar herbivory		
Tissue	Compound	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Mature leaf	MANOVA	4, 33	6.61	<0.001	4, 33	0.68	0.61	4, 33	4.22	0.008
	Hemigossypolone	1, 36	12.37	0.001	1, 36	1.06	0.30	1, 36	2.09	0.16
	Gossypol	1, 36	27.07	<0.001	1, 36	0.01	0.97	1, 36	0.08	0.77
	Heliocides 1 and 4	1, 36	10.38	0.002	1, 36	1.05	0.30	1, 36	15.31	<0.001
	Heliocides 2 and 3	1, 36	12.98	<0.001	1, 36	0.14	0.70	1, 36	3.04	0.08
Immature leaf	MANOVA	4, 33	3.97	0.01	4, 33	29.11	<0.001	4, 33	6.14	<0.001
	Hemigossypolone	1, 36	0.37	0.55	1, 36	44.72	<0.001	1, 36	1.43	0.24
	Gossypol	1, 36	0.29	0.57	1, 36	45.23	<0.001	1, 36	0.39	0.53
	Heliocides 1 and 4	1, 36	10.51	0.002	1, 36	116.61	<0.001	1, 36	17.18	<0.001
	Heliocides 2 and 3	1, 36	5.96	0.02	1, 36	89.57	<0.001	1, 36	8.46	0.006
Root	MANOVA	5, 32	19.40	<0.001	5, 32	0.95	0.46	5, 32	1.80	0.14
	Hemigossypol	1, 36	50.99	<0.001	1, 36	2.17	0.14	1, 36	4.23	0.047
	Methoxyhemigossypol	1, 36	97.31	<0.001	1, 36	0.84	0.37	1, 36	5.37	0.026
	Gossypol	1, 36	7.71	0.008	1, 36	0.73	0.40	1, 36	1.81	0.19
	Monomethylgossypol-ether	1, 36	11.94	0.001	1, 36	0.008	0.93	1, 36	0.96	0.33
	Dimethylgossypol-ether	1, 36	13.66	<0.001	1, 36	0.23	0.62	1, 36	0.37	0.54

total terpenoid levels increased 4.2-fold in plants exposed to root herbivory relative to control plants. Terpenoid levels were not influenced by above-ground herbivory, while levels in plants with root and foliar herbivory increased 2.3-fold relative to control plants (Table 1, Figure 2). In immature leaves, root herbivory resulted in 2.3-fold increases in terpenoid levels relative to control plants, whereas plants

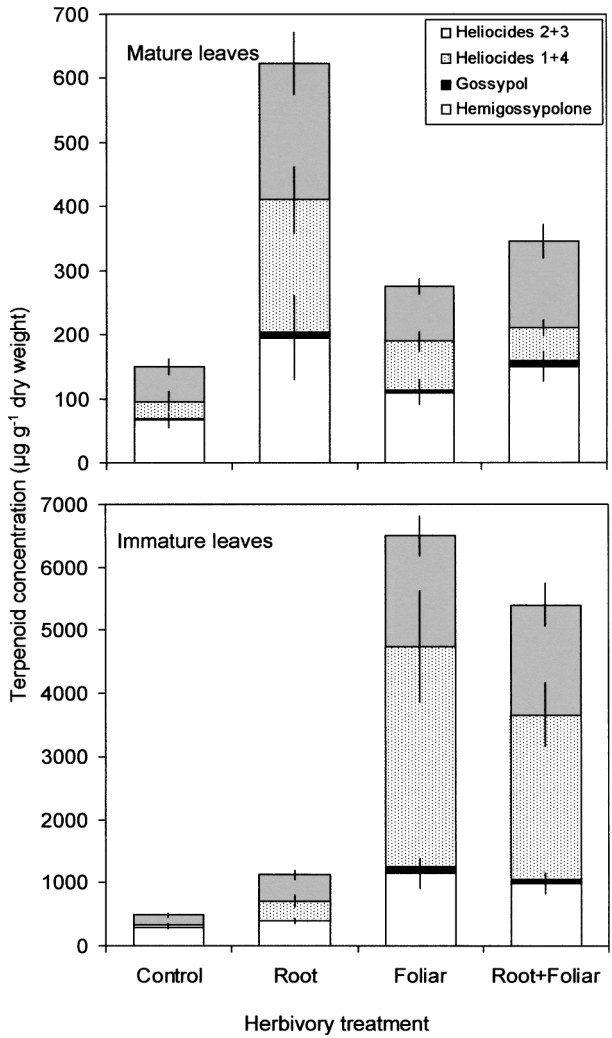

FIG. 2. Mean (±SE) terpenoid aldehyde content in mature and immature leaf tissue of cotton plants exposed to different herbivory treatments.

TABLE 2. MULTIVARIATE TEST OF THE INFLUENCE OF LEAF TYPE AND HERBIVORY TYPE ON FOLIAR TERPENOID ALDEHYDE LEVELS

Treatment	df	*F*	*P*
Leaf	4, 69	60.09	<0.001
Root herbivory (RH)	4, 69	5.55	<0.001
Foliar herbivory (FH)	4, 69	15.73	<0.001
Leaf × RH	4, 69	1.73	0.15
Leaf × FH	4, 69	10.06	<0.001
RH × FH	4, 69	9.07	<0.001
Leaf × RH × FH	4, 69	0.41	0.79

Note. Results from MANOVA based on Wilks' test.

subjected to foliar herbivory showed a 13.5-fold increase in immature leaves. Levels in immature leaves in the combined treatment increased 11.2 times (Figure 2). This resulted in a significant interaction between root and foliar herbivory in both mature and immature leaves (MANOVA; Table 1). The induction in immature leaves following foliar herbivory resulted in a significant interaction between leaf type and foliar herbivory (MANOVA, $F_{4,69} = 10.06$, $P < 0.001$; Table 2). Root herbivory resulted in a more even distribution of terpenoids between mature and immature leaves. There was no significant interaction between leaf age and root herbivory (MANOVA, $F_{4,69} = 1.73$, $P = 0.15$; Table 2). In plants subjected to herbivory, the concentration of heliocides 1 and 4 increased more than concentrations of heliocides 2 and 3, resulting in a significant shift in the ratio between these compounds (Table 3). In immature leaves on plants exposed to herbivory, the ratio between hemigossypolone and heliocides decreased, but no such effect was found in mature leaves (Table 3).

TABLE 3. RATIOS BETWEEN TERPENOID ALDEHYDES IN COTTON FOLIAGE

	Heliocides (1 + 4) : Heliocides (2 + 3)		Hemigossypolone:Heliocides (1 − 4)	
Treatment	Mature leaf	Immature leaf	Mature leaf	Immature leaf
Control	0.4 ± 0.2a	0.3 ± 0.1a	1.2 ± 0.2	2.1 ± 0.4a
Root herbivory	1.0 ± 0.2b	0.8 ± 0.2b	0.5 ± 0.07	0.6 ± 0.08b
Foliar herbivory	0.9 ± 0.2b	1.9 ± 0.1c	1.1 ± 0.4	0.2 ± 0.04b
Root + foliar herbivory	0.4 ± 0.09a	1.5 ± 0.2c	0.9 ± 0.1	0.3 ± 0.04b
ANOVA	$F_{3,36} = 4.36$ $P < 0.01$	$F_{3,36} = 31.41$ $P < 0.001$	$F_{3,36} = 2.58$ $P = 0.06$	$F_{3,36} = 16.66$ $P < 0.001$

Note. Values are mean ± standard error, within columns, means with identical letters are not significantly different ($P < 0.05$) based on ANOVA followed by Tukey's HSD.

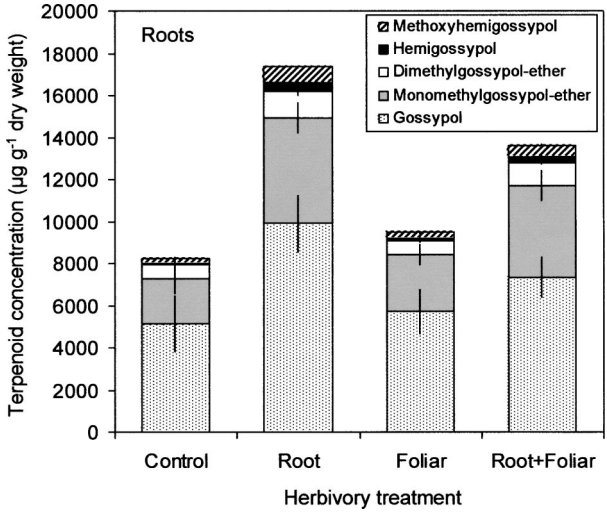

FIG. 3. Terpenoid aldehyde content in root tissue of cotton plants exposed to different herbivory treatments.

Root herbivory caused a significant increase in terpenoid concentrations in root tissue, but no significant effects were found in roots of plants exposed to foliar herbivory (Figure 3; Table 1). In plants exposed to the combination of root and foliar herbivory, total terpenoid concentrations were significantly higher (1.7 times) than in control plants but the effect was not as strong as for plants exposed to root herbivory only (2.1 times, Figure 3). In plants exposed to root herbivory, either alone or in combination with foliar herbivory, the relative terpenoid induction was highest for hemigossypol (4.0 times increase) followed by methoxyhemigossypol (3.3 times increase). Gossypol showed the largest absolute increase (2200–4700 $\mu g \cdot g^{-1}$ dry weight), but relative levels of gossypol in root tissue increased only 1.4–1.9 times in response to root herbivory.

Effects of Mechanical Root Damage on Terpenoid Accumulation. Mechanical root damage caused a significant increase in terpenoid accumulation above- and below-ground (Table 4). MANOVA indicated that these results were significant only in immature leaves (MANOVA immature leaves: $F_{8,26} = 3.62$, $P = 0.005$; mature leaves: $F_{8,26} = 0.95$, $P = 0.49$; Root: $F_{10,24} = 1.65$, $P = 0.15$). In immature leaves, mechanical root damage resulted in significant increases in levels of heliocides 1 and 4 relative to control plants. Below-ground, concentrations of methoxyhemigossypol increased significantly following mechanical root damage, but the level of increase was relatively low (Table 4). Concentrations of terpenoids in plants with foliar herbivory also increased in immature leaves, but only hemigossypolone levels were significantly higher than control plants (Table 4).

TABLE 4. TERPENOID ALDEHYDE ACCUMULATION FOLLOWING MECHANICAL ROOT DAMAGE OR FOLIAR HERBIVORY BY *S. exigua*

Tissue	Compound	Control	Mechanical root damage	Foliar damage	df[a]	F[a]	P[a]
Mature/ damaged leaf	Hemigossypolone	322 ± 41	233 ± 32	256 ± 33	2,16	1.54	0.24
	Gossypol	10 ± 4	5 ± 3	15 ± 11	2,16	0.57	0.59
	Heliocides 1 and 4	59 ± 26	65 ± 15	45 ± 11	2,16	0.44	0.64
	Heliocides 2 and 3	231 ± 34	206 ± 32	203 ± 22	2,16	0.25	0.78
Immature/ undamaged leaf	Hemigossypolone	825 ± 134a	1115 ± 137a	1772 ± 172b	2,16	9.79	0.001
	Gossypol	23 ± 10	51 ± 11	49 ± 15	2,16	1.21	0.32
	Heliocides 1 and 4	163 ± 74a	458 ± 76b	368 ± 58ab	2,16	4.21	0.03
	Heliocides 2 and 3	389 ± 63	577 ± 77	658 ± 73	2,16	3.11	0.07
Root	Hemigossypol	1060 ± 22	1200 ± 49	1154 ± 36	2,16	2.79	0.09
	Methoxyhemigossypol	1194 ± 31a	1327 ± 43b	1233 ± 14ab	2,16	4.33	0.03
	Gossypol	10717 ± 1800	8177 ± 1043	8304 ± 986	2,16	1.20	0.33
	Monomethylgossypol-ether	4265 ± 755	4641 ± 821	3304 ± 454	2,16	1.02	0.38
	Dimethylgossypol-ether	1573 ± 103	1644 ± 170	1463 ± 96	2,16	0.50	0.60

Note. Values are mean ± standard error (μg g^{-1} dry weight), within rows means with identical letters are not significantly different ($P < 0.05$) based on ANOVA followed by Tukey's HSD.

[a] Results of one-way ANOVA.

DISCUSSION

In agreement with optimal defense allocation theory, we found that young, immature leaves of cotton plants contained considerably higher levels of terpenoid aldehydes than older leaves. Feeding by *S. exigua* for 2 days on *G. herbaceum* caused no significant changes in quality or quantity of terpenoid aldehydes in older, mature leaves, but we found profound differences in young leaves developing on plants exposed to foliar feeders compared to young leaves on control plants. A number of studies have shown that foliar terpenoid aldehyde concentrations are enhanced in cotton plants that are damaged either mechanically or by foliar feeding insects such as *Spodoptera* larvae (McAuslane et al., 1997; McAuslane and Alborn, 1998; Anderson and Alborn, 1999). Although we did not observe a local increase in terpenoid aldehydes in the leaf that was exposed to herbivory, we did observe a significant increase of hemigossypolone and the heliocides 1 and 4 in the side leaves adjoining the damaged leaf. These side leaves are much younger in age. These results, therefore, support the finding that terpenoid aldehydes are directed to the youngest, most valuable leaves (Zangerl and Bazzaz, 1992). The allocation of (inducible) defenses within plants is thought to be associated with the value of the plant part to plant fitness (Krischik and Denno, 1983). Young leaves are thought to be more valuable to the plant than old leaves, because their residual lifetime photosynthetic capacity is much larger (van Dam et al., 1996).

The systemic induction of terpenoids in younger leaves differs from the induction of extrafloral nectaries in cotton, which allow the plant to recruit ants as protective agents. Upon herbivore attack, *G. herbaceum* increases extrafloral nectar production by a factor of 10 (Wäckers et al., 2001). In contrast to the pattern of direct defense induction, the induced increase in extrafloral nectar secretion is primarily restricted to the damaged leaf. This pattern of nectar induction is thought to enable plants to actively guide ants to the site of herbivore attack (Wäckers et al., 2001; Rudgers, 2003). Young leaves appear to be protected against herbivory via induced direct defense even when the attack is restricted to older leaves, while indirect defense via extrafloral nectar that attracts the natural enemy of the herbivore is directed specifically towards the leaf under attack. Other studies have shown that cotton leaves are also protected indirectly by systemic release of herbivore-induced volatiles that attract parasitic wasps (Röse et al., 1998).

The major aldehyde in mature leaves of *G. hirsutum* is hemigossypolone, while in immature buds gossypol is dominant (Stipanovic et al., 1988). Hemigossypolone and gossypol are both formed from hemigossypol, but in leaves and other tissues exposed to light, conversion to hemigossypolone is more prevalent than conversion to gossypol (Liu et al., 1999). Similarly, we recorded hemigossypolone as the predominant aldehyde in *G. herbaceum* foliage, with low quantities of gossypol. The finding that gossypol was the only terpenoid aldehyde in *G. herbaceum*

cotyledons may, in part, be explained by the fact that this is the sole terpenoid aldehyde in cotton seeds (Stipanovic et al., 1988).

Aboveground herbivory did not result in an increase in root terpenoid aldehyde concentrations. Other studies have shown that terpenoid concentrations in the roots of cotton are enhanced following below-ground feeding by nematodes or infection by bacterial or fungal diseases (Mace et al., 1990; Koshkoo et al., 1993; Zhang et al., 1993; Howell et al., 2000). A functional explanation for our finding might be that damage signals coming from foliage indicate imminent danger for young leaves but not for roots. Root damage, in contrast, did increase the levels of terpenoid aldehydes in both roots and shoots. Given that we can expect physiological limitations to the production of terpenoid aldehydes, this presents a potential conflict between root and foliar defense. In the treatment where root and foliar damage were applied simultaneously, this conflict was manifested in reduced terpenoid aldehyde levels in both compartments, as compared to the levels following either root or foliar damage.

While foliar herbivory resulted in a localized increase of terpenoid aldehydes in immature leaves, foliage of plants exposed to root herbivory also showed increased levels of aldehydes in mature leaves. There were no major shifts in relative concentrations of terpenoid aldehydes produced following above- or below-ground herbivory. However, it appears that depending on the type of herbivory, aldehydes are allocated differently between above- and below-ground tissues, suggesting that these aldehydes are present in the plant in limiting quantities. Aldehydes produced in the root tissue can be either allocated above- or below-ground (Liu et al., 1999). The primary root-produced terpenoid, hemigossypol, can be dimerized via a peroxidase to gossypol and allocated primarily below-ground or oxidized to hemigossypolone and allocated above-ground (Bell et al., 1978; Liu et al., 1999). Plants that are exposed to both above- and below-ground herbivory appear to primarily allocate aldehydes above-ground. This likely has repercussions for below-ground defense. In plants exposed to both root and foliar herbivory, aldehyde quantities in root tissue were roughly half that of plants exposed to root herbivory only.

Although root herbivory has been largely overlooked, this study in congruence with others (e.g., Hanounik and Osborne, 1977) shows that it can result in enhanced concentrations of allelochemicals above-ground. Here, we have shown that the specific terpenoid aldehydes in foliage that are enhanced in quantity following foliar damage are also enhanced when plants are exposed to root damage. However, we also show that there are marked differences between both types of damage with regard to the spatial distribution of the induced foliar terpenoids. We previously reported that extrafloral nectar production in *G. herbaceum* also increased following either root herbivory or mechanical root damage (Wäckers and Bezemer, 2003) and that root herbivory reduced the growth rate and food consumption of *S. exigua* larvae feeding above-ground (Bezemer et al., 2003). The current results emphasize that below-ground herbivores can influence above-ground

processes (de Deyn et al., 2003; van der Putten, 2003) and that root feeders cannot be ignored when studying insect–plant interactions.

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QUALITATIVE AND QUANTITATIVE VARIATION AMONG VOLATILE PROFILES INDUCED BY *Tetranychus urticae* FEEDING ON PLANTS FROM VARIOUS FAMILIES

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Abstract—Many plant species are known to emit herbivore-induced volatiles in response to herbivory. The spider mite *Tetranychus urticae* Koch is a generalist that can feed on several hundreds of host plant species. Volatiles emitted by *T. urticae*-infested plants of 11 species were compared: soybean (*Glycine max*), golden chain (*Laburnum anagyroides*), black locust (*Robinia pseudo-acacia*), cowpea (*Vigna unguiculata*), tobacco (*Nicotiana tabacum*), eggplant (*Solanum melalonga*), thorn apple (*Datura stramonium*), sweet pepper (*Capsicum annuum*), hop (*Humulus lupulus*), grapevine (*Vitis vinifera*), and ginkgo (*Ginkgo biloba*). The degree to which the plant species produced novel compounds was analyzed when compared to the odors of mechanically damaged leaves. Almost all of the investigated plant species produced novel compounds that dominated the volatile blend, such as methyl salicylate, terpenes, oximes, and nitriles. Only spider mite-infested eggplant and tobacco emitted a blend that was merely quantitatively different from the blend emitted by mechanically damaged or clean leaves. We hypothesized that plant species with a low degree of direct defense would produce more novel compounds. However, although plant species with a low direct defense level do use indirect defense to defend themselves, they do not always emit novel compounds. Plant species with a high level of direct defense seem to invest in the production of novel compounds. When plant species of the Fabaceae were compared to plant species of the Solanaceae, qualitative

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differences in spider mite-induced volatile blends seemed to be more prominent in the Fabaceae than in the Solanaceae.

Key Words—Headspace analysis, variation, Fabaceae, Solanaceae, specificity, herbivore-induced plant volatiles, mites, *Phytoseiulus persimilis*, methyl salicylate.

INTRODUCTION

Semiochemicals (infochemicals) are important mediators of tritrophic interactions such as predator–herbivore–plant interactions or parasitoid–herbivore–plant interactions. Behavioral and chemical studies have proven that carnivorous arthropods, such as predators and parasitoids, can be attracted to plant volatiles that are induced by their prey or hosts (for example: Agelopoulos and Keller, 1994; Blaakmeer et al., 1994; Turlings et al., 1995; Dicke et al., 1998; Du et al., 1998; Dicke, 1999a; Shiojiri et al., 2001). Moreover, carnivores are often not, or to a lesser extent, attracted to volatiles from mechanically damaged leaves, or to volatiles from leaves infested by herbivores that are not suitable as prey or hosts (Sabelis and van de Baan, 1983; de Moraes et al., 1998; Du et al., 1998; Dicke, 1999a; Guerrieri et al., 1999; Shimoda and Dicke, 2000). Infochemicals involved in such interactions are called synomones, which means that they are beneficial for both emitter and receiver (Nordlund and Lewis, 1976; Dicke and Sabelis, 1988).

Herbivore-induced volatile profile can differ qualitatively or quantitatively from the profile emitted in response to mechanical damage. Dicke et al. (1998) discriminated two types of volatile profiles emitted from herbivore-infested plants. The first type was qualitatively different from the blend emitted by mechanically damaged leaves, with the emission of novel compounds that make a major contribution to the total blend. The emission of novel compounds in the qualitatively different volatile blend represents induction of novel biosynthetic pathways (Paré and Tumlinson, 1997; Dicke, 1999b). This can be considered a more sophisticated form of indirect defense than the emission of volatiles through pathways that are also induced by mechanical damage. The second type of volatile profile is one that is quantitatively different from that emitted by mechanically damaged leaves. In herbivore-infested leaves, larger quantities of compounds are emitted that are also produced by mechanically damaged leaves. In addition, compounds are emitted from the herbivore-infested plant for a longer time than from mechanically damaged plants (Steinberg et al., 1993).

Plant species that have a strong direct defense may not invest in indirect defense through the emission of specific volatiles. They might induce more compounds that contribute to the plant's direct defense and, simultaneously, quantitatively increase their volatile emission profile and attract carnivorous arthropods. Plant species that do not have a strong direct defense may rely on an effective

indirect defense through specific volatiles that provide carnivores with reliable information on herbivore presence and identity. It is not known to what degree these two different mechanisms are compatible and to what extent a plant can bear the costs of using both types. A tradeoff between the level of direct and indirect plant defense has been shown for *Nicotiana attenuata* Torr. Ex. Watson (Kahl et al., 2000). When *Manduca sexta* (Lepidoptera: Sphingidae) larvae feed on *N. attenuata* these plants increase the release of volatile terpenoids (indirect defense), but do not increase levels of nicotine (direct defense). In contrast, both types of defense are induced simultaneously in tomato, albeit the induced indirect defense does not consist of major novel compounds (Dicke et al., 1998; Thaler et al., 2002).

For the spider mite *Tetranychus urticae* and its natural enemy, the predatory mite *Phytoseiulus persimilis*, it has been shown that the predatory mite is attracted by spider mite-induced compounds in volatile blends of several plant species. Investigated plant species include lima bean (Dicke et al., 1990), apple (Takabayashi et al., 1991), cucumber (Takabayashi et al., 1994b), tomato (Dicke et al., 1998), and gerbera (Krips et al., 1999). Both lima bean and cucumber emit a qualitatively different blend in response to spider mite damage compared to mechanical damage (Dicke et al., 1990; Takabayashi et al., 1994b). Tomato emits a blend in response to spider mite feeding that is only quantitatively different from the blend of mechanically damaged plants; there are no novel volatiles emitted from tomato after spider mite damage that dominate the blend (Dicke et al., 1998).

The degree of direct defense of several plant species from various families to the spider mite *T. urticae* has been investigated (Van Den Boom et al., 2003), as well as their capability to attract the spider mite's natural enemy *P. persimilis* (Van Den Boom et al., 2002). To obtain more information on the mechanism of the induced indirect defense, the identity of the volatiles that are released by plants from various species when *T. urticae* is feeding on these plants was investigated and compared to the volatiles emitted from uninfested and mechanically damaged leaves. In this way, it can be assessed whether major novel biosynthetic pathways are induced by the spider mite's feeding. Special attention was given to species of the Fabaceae and the Solanaceae. These families vary in their degree of direct defense (Van Den Boom et al., 2003). All investigated species of the Fabaceae were readily accepted for feeding by the spider mite *T. urticae*, while those of the Solanaceae showed more variation, from being well accepted to poorly accepted.

METHODS AND MATERIALS

Plant Material. The plant species used are listed in Table 1. Plants were grown in a greenhouse (20–30°C, RH 60–80%, and 16L:8D photoregime). Lima bean plants (*Phaseolus lunatus* L.) that were used as host plants for the spider

TABLE 1. PLANTS AND TREES USED IN THE HEADSPACE EXPERIMENTS

Family	Genus	Species	Common name	Cultivar
Fabaceae	*Glycine*	*max*	Soybean	Gieso
Fabaceae	*Laburnum*	*anagyroides*	golden chain	
Fabaceae	*Robinia*	*pseudo-acacia*	black locust	
Fabaceae	*Vigna*	*unguiculata*	cowpea	Black eye
Ginkgoaceae	*Ginkgo*	*biloba*	ginkgo	
Moraceae	*Humulus*	*lupulus*	hop	
Solanaceae	*Capsicum*	*annuum*	sweet pepper	Lambada
Solanaceae	*Datura*	*stramonium*	thorn apple	
Solanaceae	*Nicotiana*	*tabacum*	tobacco	SR1
Solanaceae	*Solanum*	*melalonga*	eggplant	Black beauty
Vitaceae	*Vitis*	*vinifera*	grapevine	Glorie van Boskoop

mites were reared under the same conditions. Hops were vegetatively propagated from plants grown outdoors and subsequently reared in the greenhouse. Grapevine (height: about 0.5–1 m), black locust trees (height: 1–1.5 m), and golden chain trees (height: 1–1.5 m) were grown outdoors in the backyard of the greenhouse of Wageningen University. Trees were watered automatically at regular intervals. Leaves of a ginkgo tree (height: about 15–20 m) were taken in spring or summer from a tree that grew in the Arboretum of Wageningen University. Ages of the plants and trees, and their mean leaf areas are given in Table 2. Mean leaf area was measured with an area meter (Li-Cor LI-3100) and is the average of 10 leaves unless mentioned otherwise. Test plants and trees (except ginkgo) were kept per

TABLE 2. INFORMATION ON LEAVES USED IN THE HEADSPACE EXPERIMENTS

Common name	Age (weeks)	Number of leaves used	Mean leaf area ±SD (cm^2)	Spider mite damage (days)
soybean	5	2	115 ± 10	8
golden chain	—[a]	10	20 ± 5[c]	3
black locust	—[a]	50	15 ± 5[c]	4
cowpea	4	2	160 ± 25	8
ginkgo	—[a]	5	40 ± 5	—[d]
hop	25[b]	3	65 ± 5[c]	8
sweet pepper	12	3	120 ± 20	6
thorn apple	6	3	115 ± 25	8
tobacco	8	3	125 ± 35	7
eggplant	8	2	260 ± 25	7
grapevine	—[a]	2	165 ± 20	8

[a] Age not known, but $\geq$ 3 years.
[b] Age calculated from the time the plant was vegetatively propagated.
[c] An average of 50 leaves was taken to calculate the average leaf size.
[d] No spider mite-infestation, but jasmonic acid used for volatile-induction.

species in a separate compartment in the greenhouse. They were infested by placing spider mite-infested lima bean leaves on top of their leaves. Only fully expanded leaves were used for the experiments. In the case of soybean and cowpea, fully expanded trifoliar leaves were used.

Rearing of Mites. The spider mites, *Tetranychus urticae*, were reared on lima bean plants under the same conditions as the uninfested lima bean plants. This was done in a separate greenhouse compartment.

Setup for Headspace Collection. A method was used to sample the volatiles from six 5-l glass jars connected in parallel. Synthetic air (grade 5.0) was led via Teflon tubing over an activated charcoal filter and a molecular sieve (5A). Via a glass connection, the air was divided equally over the six glass jars. Air entered the jars via the top and left via a glass tube (160.0 mm long $\times$ 6.0 mm OD, 4.0-mm ID) that was connected to the air outlet at the bottom of the jar. This tube was filled with 90 ± 10 mg Tenax-TA (20–35 mesh, 500–840 μm) and kept in place by two stainless steel frits. Prior to headspace collection, Tenax tubes were cleaned in a Thermal Desorption Oven (TDS, Gerstel, The Netherlands) at 250°C. A flow meter was used to measure the flow rate at the end of the tubes. Flow rate was kept at 100 ± 20 ml/min. Fresh test plant leaves were cut and placed with their petioles into a thick layer of wet cotton wool in five of the jars, to obtain five replicate measurements. Plant volatiles were sampled for 2 hr. For most plant species, 2–3 test plant leaves were used, but more were used for ginkgo and golden chain, i.e., 5 and 10, respectively, because these leaves were much smaller than leaves of the other plant species (Table 2). For black locust, 5 small branches each with 10 leaves were used. The sixth glass jar contained only a piece of wet cotton wool, and the air sampled from this jar was used as a blank.

Besides the parallel headspace setup, one 5-l glass jar with two Tenax tubes at opposite outlets was used. Flow rate through both tubes was kept at 100 ± 20 ml/min. In this way, two replicate headspace samples from the same set of plant leaves were obtained. One tube of the two twin replicates was desorbed in a thermal desorption system (TDS) connected to an autosampler (TDS-A), and the compounds were analyzed on an RTX-200 column in a gas chromatograph (HP5890) and detected with a flame ionization detector (FID) (see below). The same procedure was followed for the other five tubes that were obtained from the parallel headspace experiment. The other tube was also desorbed in a TDS. Compounds were analyzed on the same type of column in a GC connected to a mass spectrometer (GC-MS; Varian 3400). A Finnigan MAT 95 mass spectrometer was used for detection (see below). After GC-MS analysis, compounds were identified by comparison of their retention indices and mass spectra to reference spectra in the NIST and the natural products library of the mass spectrometry section of Wageningen University. Subsequently, the GC-MS chromatogram with the identified peaks was compared with the gas chromatogram obtained from the twin-replicate Tenax tube that had been analyzed with an FID, by comparing peak

shapes and retention times. When the retention times of the peaks in the gas chromatogram were known, they were compared to retention times of the compounds in the five replicate chromatograms from the parallel headspace measurements, which were measured with the same apparatus and on the same column.

Gas Chromatography. Volatiles adsorbed on the Tenax in the headspace tube were desorbed in a splitless mode in a TDS with helium (grade 6.0). Temperature of the TDS was kept at 30°C for 0.5 min and subsequently increased at a rate of 60°C/min to 250°C. Desorbed compounds were transferred via a heated transfer line (300°C) into the empty liner of a GC (HP 5890). In the injector, volatiles were trapped again at −150°C. After trapping, the injector was heated at a rate of 12°C/sec up to 250°C. Volatiles were transferred in splitless mode onto an uncoated deactivated fused silica precolumn that was split into two columns, an RTX-200 column (Restek corporation: 60-m long, 0.25-mm ID, 0.25-μm film thickness) and a non-polar AT-1 column (Alltech: 60-m long, 0.25-mm ID, 0.25-μm film thickness). Two columns of different polarity were chosen to obtain quantitative reference values in order to resolve peaks that coeluted. The GC oven temperature was first kept for 3 min at 40°C and then increased with a rate of 4°C/min to 240°C. An FID was used to detect the volatile compounds. Chromatograms were obtained for five headspace collections of each treatment and one blank.

Gas Chromatography and Mass Spectrometry. A gas chromatograph (Varian 3400) connected to a Finnigan MAT 95 mass spectrometer was used to identify the headspace compounds collected on the Tenax. Volatiles were thermally desorbed in splitless mode with helium as carrier gas at a temperature of 250°C and subsequently cryofocused in a Thermal Desorption Cold Trap Injector (M-16200, Chrompack, The Netherlands) at a temperature of −90°C. The Cold Trap Injector was ballistically heated to 220°C, and the volatiles were transferred in a splitless mode onto an RTX-200 column (Restek: 60-m long, 0.25-mm ID, 0.25-μm film thickness). The oven temperature was kept for 2 min at 40°C and increased at a rate of 4°C/min to 250°C. The MS was operated in the 70 eV EI ionization mode. Spectra were continuously scanned in a mass range from 24 to 300 amu at a rate of 0.5 sec/decade.

Leaf Treatments. For each test plant species, spider mite-infested, mechanically damaged, and clean leaves were used for headspace analyses. Headspace was collected from each treatment in five replicates for each test plant species. After each collection, the jars were washed, and dried at 110°C. Leaves were freshly cut from the plant, and headspace was collected immediately. Spider mite-infested leaves were obtained from plants on which spider mites had been feeding for 3–8 days (Table 2). Leaves were mechanically damaged with carborundum on a wet cotton wool pad after cutting the leaf, and headspace was collected just after inflicting the damage. On each leaf, a damaged spot of about 1 × 1-cm was created.

It was not possible to rear spider mites on ginkgo, because ginkgo was not accepted as a host plant by *T. urticae*. Therefore, these leaves were treated with

jasmonic acid to simulate spider mite infestation (Boland et al., 1995; Dicke et al., 1999). For that purpose, 5 sets of 5 ginkgo leaves were placed with their petioles into vials with 10 ml of an aqueous jasmonic acid solution (1 mM) for 1 day. The vials were sealed with parafilm. Uptake of jasmonic acid solution by ginkgo leaves was 1–2 ml after 1 day. As a control, 5 sets of 5 leaves were placed for 1 day in vials with 10 ml of a diluted acidic solution (1 mM HCl).

Statistics. The total amounts of volatiles produced by noninfested, mechanically damaged, and spider mite-infested leaves were tested with the Kruskal Wallis test, followed by a multiple comparison test. Relative amounts of compounds were compared among the different treatments of the same test plant species by using the Kruskal Wallis test followed by a multiple comparison test.

RESULTS

Compounds that represented at least 0.5% of the total amount of the volatile blend emitted by clean, mechanically damaged or spider mite-infested plant leaves are depicted in Figure 1 (panels a–k). Total amounts of volatiles produced by the plant leaves with statistical differences are presented in Table 3.

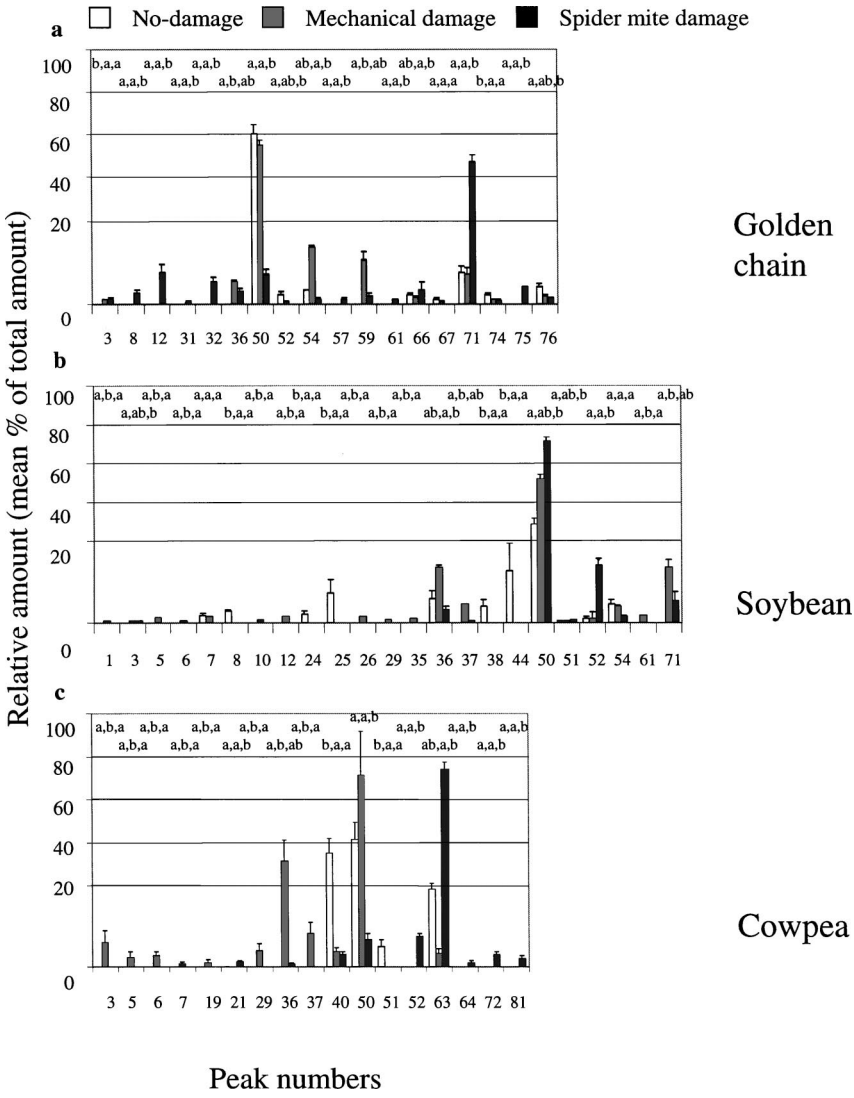
Spider Mite-Induced Compounds of Fabaceae Plants. When infested by the spider mite, all fabaceous species produced compounds that were either not released or released only in small amounts by mechanically damaged or clean leaves (Figures 1a–d). Additionally, they all released the green leaf volatiles (Z)-3-hexen-1-ol (36) and (Z)-3-hexen-1-ol, acetate (50) when mechanically damaged. These volatiles were also emitted when plants were infested by spider mites, but in lower proportions.

*Golden Chain (*Laburnum anagyroides*).* Spider mite-infested leaves from golden chain emitted (*E*,*E*)-α-farnesene (71) as the dominant compound in the blend. This compound is present in significantly larger proportions in the headspace from mite-infested leaves than in the headspace from mechanically damaged and clean leaves: 47%, 7%, and 8%, respectively. This means that in absolute amounts (*E*,*E*)-α-farnesene (71) is emitted in seventy-fold larger amounts from mite-infested leaves than from mechanically damaged leaves. Additionally, novel compounds are present in the spider mite-induced blend. The compounds that are most abundant include 2-methylbutanenitrile (8) (3%), (syn) or (anti)-2-methylbutanal oxime (12) (8%), 3-methyl-1-butanol (32) (5%), and α-humulene (75) (4%). The green leaf compound (*Z*)-3-hexen-1-ol, butanoate (54) is present in a larger proportion in the blend from mechanically damaged leaves (14%) than in the blend from mite-infested leaves (1%). However, in absolute quantities, the amount emitted from mechanically damaged leaves is similar to that of mite-infested leaves (104 vs. 107 peak area units).

Soybean (Glycine max). Spider mite-infested soybean leaves emitted the green leaf volatile 3-hexen-1-ol, acetate (50) and methyl salicylate (52) as dominant compounds (72% and 13%, respectively). (*Z*)-3-Hexen-1-ol, acetate (50) was emitted in large absolute quantities of about 13,400 peak area units. The relative percentage of methyl salicylate was statistically different from the percentage in the headspace of mechanically damaged leaves. In absolute amounts, more than 50 times as much methyl salicylate is produced by spider mite-infested than by mechanically damaged soybean leaves. (*E*,*E*)-α-Farnesene (71) was emitted in relatively lower proportions by mite-infested than by mechanically damaged soybean leaves (5% and 12%, respectively), but in absolute amounts, twice as much

FIG. 1. a–k. Plant compounds present at 0.5% or higher proportions in the headspace of clean leaves, mechanically damaged leaves, and spider mite-infested leaves, are depicted. For ginkgo, the headspaces of clean leaves, HCl-treated leaves and JA-treated leaves are depicted. The mean relative amounts are presented with the standard error ($\pm$SE). The mean total amount ($\pm$SE) of plant volatiles in area counts as measured by gas chromatography with the use of an FID is depicted in the figures as well. Bars for the same compound that are labeled with the same letter are not statistically significant (Kruskal Wallis test, $\alpha = 0.05$). Compounds depicted in the figures are: *Aldehydes*: 1. (*Z*)-2-pentenal 2. (*E*,*E*) or (*E*,*Z*) 2,4-hexadienal 3. (*E*)-2-hexenal 4. (*Z*)-2-hexenal 5. (*Z*)-3-hexenal 6. hexanal 7. (*E*)-4-oxo-2-hexenal *Nitrogen containing compounds*: 8. 2-methylbutanenitrile 9. 3-methylbutanenitrile 10. 2-methylpropanal, oxime 11. 2-methylpropanal, O-methyloxime 12. (syn) or (anti)-2-methylbutanal, oxime 13. (anti) or (syn)-2-methylbutanal, oxime 14. (syn) or (anti)-3-methylbutanal, oxime 15. (anti) or (syn)-3-methylbutanal, oxime 16. 2-methylbutanal, O-methyloxime 17. 3-methylbutanal, O-methyloxime 18. benzeneacetonitrile 19. indole 20. phenylacetaldehyde, O-methyloxime (tentative) *Ketones*: 21. 2-butanone 22. 1-penten-3-one 23. 3-pentanone 24. 4-methyl-3-penten-2-one 25. 4-hydroxy-4-methyl-2-pentanone 26. 1-octen-3-one 27. (*Z*)-jasmone *Alcohols—nonterpenoid*: 28. 2-methyl-1-propanol 29. 1-penten-3-ol 30. 2-penten-1-ol 31. 2-methyl-1-butanol 32. 3-methyl-1-butanol 33. 1-pentanol 34. 3-pentanol 35. (*E*)-2-hexen-1-ol 36. (*Z*)-3-hexen-1-ol 37. 1-hexanol 38. 2,4-pentanediol, 2-methyl 39. 2-phenylethanol 40. 1-octen-3-ol 41. 3-ethyl-4-methylpentanol 42. eugenol *Alcohols—terpenoid*: 43. linalool *Carboxylic acids*: 44. hexanoic acid *Esters*: 45. 2-methylbutanoic acid, methylester 46. (E)-2-hexenoic acid, methylester 47. methyl benzoate 48. 3-cyclohexen-1-ol, acetate 49. (*E*)-2-hexen-1-ol, acetate 50. (*Z*)-3-hexen-1-ol, acetate 51. hexyl acetate 52. methyl salicylate 53. (*E*)-2-hexen-1-ol, butanoate 54. (*Z*)-3-hexen-1-ol, butanoate 55. hexyl butanoate 56. (*Z*)-3-hexen-1-ol, tiglate 57. (*Z*)-3-hexen-1-ol, 2-methylbutanoate 58. (*Z*)-3-hexen-1-ol, hexanoate *Hydrocarbons—nonterpenoid*: 59. 2-methylheptane (tent.) *Hydrocarbons – terpenoid*: 60. limonene 61. (*E*)-β-ocimene 62. α-pinene 63. (3*E*)-4,8-dimethyl-1,3,7-nonatriene 64. (3*Z*)-4,8-dimethyl-1,3,7-nonatriene 65. α-bergamotene 66. β-caryophyllene 67. α-copaene 68. (*E*)- β-elemene 69. (*Z*)-β-elemene 70. (*Z*,*E*) or (*E*,*Z*)-α-farnesene 71. (*E*,*E*)-α-farnesene 72. (*E*)-β-farnesene 73. germacrene A 74. germacrene D 75. α-humulene 76. γ-muurolene 77. α-selinene 78. β-selinene 79. γ-selinene 80. (3*E*,7*Z*) or (3*Z*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene 81. (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene *Ethers*: 82. caryophyllene oxide.

(*E*,*E*)-α-farnesene (71) was produced by spider mite-infested leaves. The compound (*Z*)-3-hexen-1-ol (36) was emitted in higher proportions from mechanically damaged leaves (12%) than from mite-infested leaves (3%). In absolute quantities, the amount emitted from mechanically damaged leaves is on the same order or magnitude as the amount emitted from mite-infested leaves (489 vs. 542 peak area units, respectively).



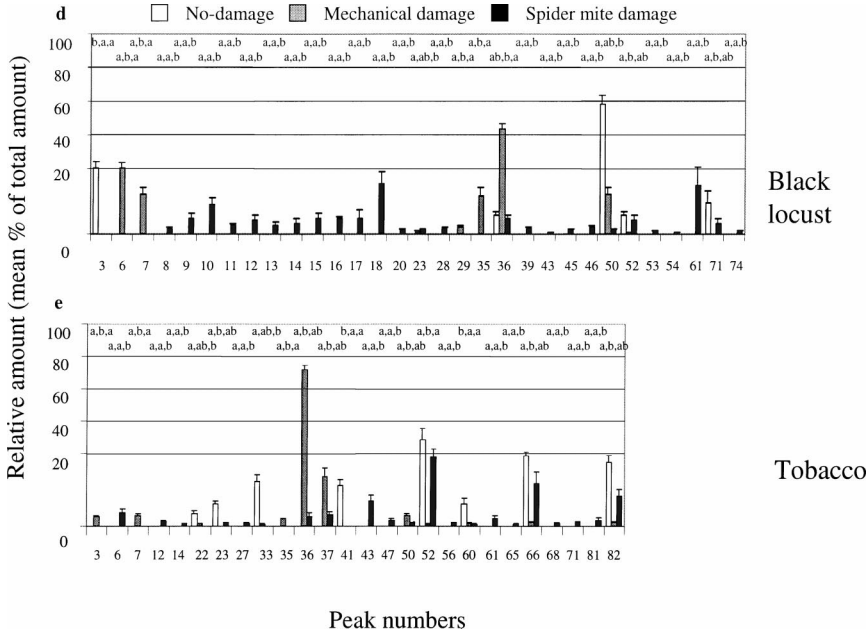


FIG. 1. (CONTINUED).

*Cowpea (*Vigna unguiculata*).* Cowpea showed an abundant production of (3*E*)-4,8-dimethyl-1,3,7-nonatriene (63) (74% of the total amount of volatiles) in response to spider mite damage. This percentage is statistically different from the percentage in the headspace of mechanically damaged leaves in both relative and absolute amounts. Additionally, methyl salicylate (52) and (*E*)-β-farnesene (72) (7% and 3%, respectively) and some minor compounds were emitted as novel compounds by the mite-infested cowpea leaves.

Black Locust (Robinia pseudo-acacia). Black locust emitted several novel nitrogen-containing compounds that dominated the blend, such as three nitriles (8, 9, 18) and nine (O-methyl)-oximes (10–17, 20). Furthermore, (*E*)-β-ocimene (61) (15%) and some other minor novel compounds were emitted as novel compounds by spider mite-infested leaves.

Spider Mite-Induced Compounds of Solanaceae Plants. Two solanaceous species, sweet pepper and thorn apple, produced large amounts of novel compounds when infested by spider mites. The other two plant species, eggplant and tobacco, produced large amounts of compounds that were also released by plants without damage or by mechanically damaged plants (Figure 1e–h). Besides nonspecific compounds, the headspace of mite-infested tobacco leaves also showed minor quantities of novel compounds. All plants released the green leaf

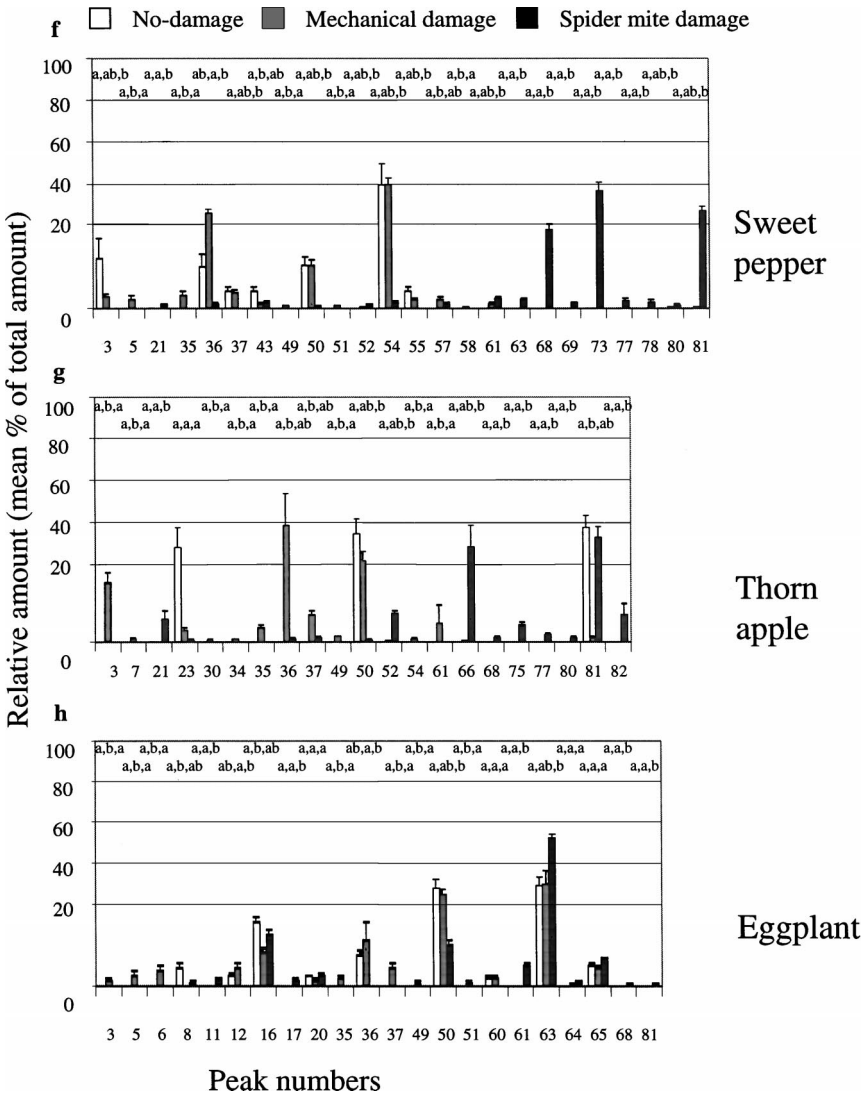


FIG. 1. (CONTINUED).

volatiles (*Z*)-3-hexen-1-ol (36) and (*Z*)-3-hexen-1-ol, acetate (50) when mechanically damaged.

*Tobacco (*Nicotiana tabacum*).* Spider mite-infested tobacco leaves emitted three dominant compounds in nearly the same ratios as the three corresponding compounds of the nondamaged leaves. Percentages for mechanically damaged

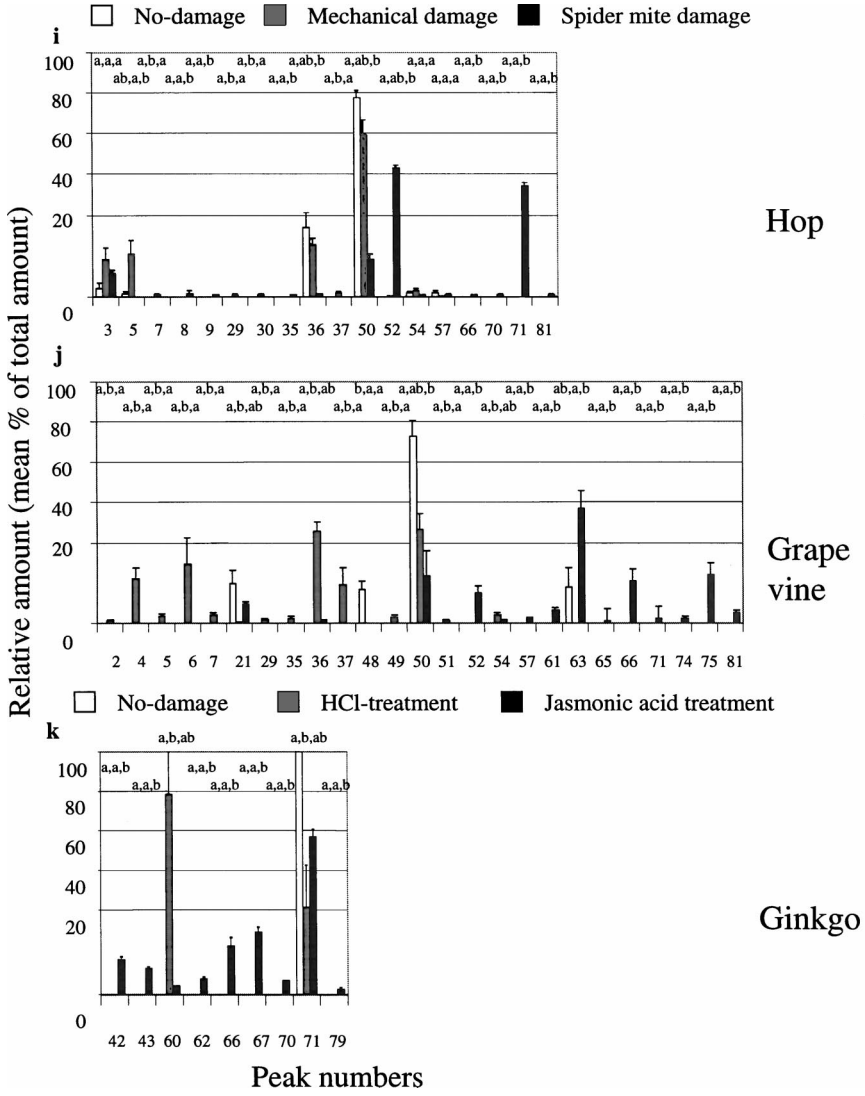


FIG. 1. (CONTINUED).

leaves are much lower, due to a high amount of (*Z*)-3-hexen-1-ol (36) (72%). The nonspecific compounds emitted by mite-infested leaves were 1-hexanol (37) (3%), methyl salicylate (52) (18%), β-caryophyllene (66) (11%), and caryophyllene oxide (82) (8%). Moreover, hexanal (6) (4%), linalool (43) (6%), and a few

TABLE 3. TOTAL AMOUNTS OF VOLATILES (MEAN AND SE; ARBITRARY PEAK AREA UNITS) EMITTED FROM PLANTS OF DIFFERENT TREATMENTS

	Treatment[a]					
	No damage		Mechanical damage		Spider mite damage	
Plant species	Mean	(SE)	Mean	(SE)	Mean	(SE)
Golden chain	415 a	(103)	748 ab	(127)	8211 b	(2194)
Soybean	1813 a	(719)	3975 ab	(1072)	18687 b	(5252)
Cowpea	113 a	(33)	2191 ab	(472)	3588 b	(1327)
Black locust	223 a	(88)	7922 ab	(2875)	15417 b	(3974)
Tobacco	135 a	(16)	3619 b	(539)	3007 b	(464)
Sweet pepper	115 a	(40)	2657 ab	(638)	11476 b	(1025)
Thorn apple	21 a	(6)	7444 b	(1573)	5861 ab	(1213)
Eggplant	520 a	(140)	779 a	(447)	6332 b	(4074)
Hop	370 a	(139)	9768 b	(1807)	5337 ab	(1255)
Grapevine	74 a	(18)	3996 ab	(1531)	8180 b	(1379)
Plant species	No treatment		HCl-treated leaves		JA-treated leaves	
Ginkgo	10 a	(3)	27 ab	(17)	757 b	(111)

[a] *Note.* For explanation of treatments and methodology see "Materials and Methods." Means in the same row that are followed by the same letter are not significantly different (Kruskal–Wallis test, $\alpha = 0.05$). Each mean value represents data from 4–5 replicates.

compounds that were produced in small quantities were emitted as novel compounds from the mite-infested leaves.

*Sweet Pepper (*Capsicum anuum*).* Spider mite-infested sweet pepper emitted three compounds in large amounts that were either not or only in small amounts emitted from mechanically damaged leaves, namely the terpenes (*E*)-β-elemene (68) (19%), germacrene A (73) (37%), and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (81) (27%). Sweet pepper also released the green leaf volatile (*Z*)-3-hexen-1-ol, butanoate (54) in large amounts from both clean and mechanically damaged leaves (both 40%). Additionally, relatively small amounts of 2-butanone (21), linalool (43), methyl salicylate (52), (*E*)-β-ocimene (61), (3*E*)-4,8-dimethyl-1,3,7-nonatriene (63), (*Z*)-β-elemene (69), α-selinene (77), and β-selinene (78) were produced by the mite-infested leaves. In absolute amounts, however, they are not negligible.

*Thorn Apple (*Datura stramonium*).* Spider mite-infested thorn apple produced five novel compounds that made up at least 5% of the total headspace blend, i.e., 2-butanone (21) (5%), methyl salicylate (52) (7%), β-caryophyllene (66) (29%), (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (81) (33%), and caryophyllene oxide (82) (7%). The compound (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (81) was also produced in large amounts by undamaged leaves. Furthermore, small amounts of the novel compounds (*E*)-β-elemene (68),

α-humulene (75), α-selinene (77), and (3*E*,7*Z*) or (3*Z*,7*E*)-4,8,12-trimethyl-1,3,7, 11-tridecatetraene (80) were emitted.

*Eggplant (*Solanum melalonga*).* Spider mite-infested eggplant leaves emitted nonspecific compounds in nearly the same ratios as clean leaves and mechanically damaged leaves. These were 2-methylbutanal-O-methyl oxime (16) (13%), (3*E*)-4,8-dimethyl-1,3,7-nonatriene (63) (52%), and α-bergamotene (65) (7%). However, in absolute amounts, the mite-infested leaves produced 10-fold larger amounts than undamaged or mechanically damaged leaves. Furthermore, small amounts of some novel compounds were emitted: 2-methylpropanal-O-methyl oxime (11), 3-methylbutanal-O-methyl oxime (17), (*E*)-β-ocimene (61), (*E*)-β-elemene (68), and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (81).

Spider Mite-Induced Compounds from Plants of Other Families. Spider mite-infested plants from species of other families each emitted two or more compounds that dominated the blend (Figure 1i–k). Both hop and grapevine released the green leaf volatiles (*Z*)-3-hexen-1-ol (36) and (*Z*)-3-hexen-1-ol, acetate (50) when mechanically damaged. Ginkgo did not release any green leaf volatiles when treated with 1 mM jasmonic acid or the control (1 mM HCl) solution.

*Hop (*Humulus lupulus*).* In hops, two major novel compounds were emitted from the spider mite-infested leaves, namely methyl salicylate (52) (43%), which is only induced in small amounts in mechanically damaged leaves, and (*E*,*E*)-α-farnesene (71) (34%). Furthermore, small amounts of 2-methylbutanenitrile (8), 3-methylbutanenitrile (9), β-caryophyllene (66), (*Z*,*E*) or (*E*,*Z*)-α-farnesene (70), and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (81) were found in the mite-infested blend.

*Grapevine (*Vitis vinifera*).* Spider mite-infested grapevine leaves released several novel compounds that dominated the blend. These are methyl salicylate (52) (8%), (3*E*)-4,8-dimethyl-1,3,7-nonatriene (63) (37%), β-caryophyllene (66) (11%), and α-humulene (75) (12%). In the headspace of mite-infested grapevine leaves, (3*E*)-4,8-dimethyl-1,3,7-nonatriene (63) occurred in 4-fold higher concentrations (37%) than in the headspace of uninfested leaves (9%). In absolute amounts, the compound was produced about 450 times more abundantly relative to uninfested leaves.

*Ginkgo (*Ginkgo biloba*).* Neither undamaged leaves, nor HCl-treated leaves of ginkgo produced many volatiles. However, when treated with 1 mM jasmonic acid-solution, large amounts were released. The most dominant compound in the blend was (*E*,*E*)-α-farnesene (71) (57%). This is also present in high concentrations in the headspace of both undamaged and HCl-treated leaves. However, the effect of jasmonic acid on the absolute amount of (*E*,*E*)-α-farnesene (71) emitted was strong. Other novel compounds emitted by JA-treated leaves were eugenol (42) (7%), linalool (43) (5%), α-pinene (62) (3%), β-caryophyllene (66) (10%), α-copaene (67) (13%), (*Z*,*E*) or (*E*,*Z*)-α-farnesene (70) (3%), and γ-selinene (79) (1%). Limonene (60) was produced in higher percentages in HCl-treated leaves

(79%) than in jasmonic acid-treated leaves (2%). In absolute amounts, limonene was present in two-fold larger amounts in the blend emitted by the HCl-treated leaves.

Variation in Release of Plant Volatiles. Uninfested plants from all plant species except thorn apple and hop released a statistically significant smaller amount of volatiles ($P < 0.05$) compared to spider mite-infested plants (Table 3). For thorn apple and hop (Table 3), the amount of volatiles from uninfested leaves was significantly smaller than the amount released from mechanically damaged leaves, but not compared to the amount released from spider mite-infested leaves. However, the amount of volatiles from spider mite-infested leaves was of the same order as the amount from mechanically damaged leaves.

DISCUSSION

Production of Synomones. Induced defenses are a common phenomenon in the plant kingdom (Karban and Baldwin, 1997). The induction of synomones in response to herbivory has been demonstrated for a large number of species (Turlings et al., 1993; Takabayashi et al., 1994a; Scutareanu et al., 1997; Dicke et al., 1998; Du et al., 1998; Shiojiri et al., 2001). Dicke et al. (1990) reported that *T. urticae*-infested lima bean leaves were attractive to the predatory mite *P. persimilis*. They showed that the headspace of spider mite-infested lima bean leaves contained five major novel compounds. In 1999, when sensitivity of the analytical equipment had improved, Dicke et al. showed that there were many more novel compounds emitted from spider mite-infested lima bean leaves; however, many of them were present only in small amounts. The synomones linalool, methyl salicylate, (*E*)-β-ocimene, and (3*E*)-4,8-dimethyl-1,3,7-nonatriene were attractive to *P. persimilis* when individually offered to these predatory mites in an olfactometer (Dicke et al., 1990). One or more of the synomone components reported by Dicke et al. (1990) were also induced in the spider mite-infested test plant species investigated in the present study, except for golden chain. The biosynthesis of herbivore-induced synomones is *de novo* (Paré and Tumlinson, 1997; Boland et al., 1999) and involves the induction of enzyme activity (Bouwmeester et al., 1999; Degenhardt and Gershenzon, 2000).

Methyl Salicylate. Methyl salicylate was present in *T. urticae*-infested soybean, cowpea, tobacco, and hop, as a novel or dominant compound in the blend. In black locust, sweet pepper, thorn apple, and grapevine it was also present, albeit in lower concentrations. In the literature, the presence of methyl salicylate has been shown in the blend emitted by spider mite-infested leaves of apple, tomato, gerbera, and especially lima bean plants (Takabayashi et al., 1991; Dicke et al., 1998; Krips et al., 1999). In contrast, Ozawa et al. (2000) did not record methyl salicylate in the volatile blend emitted by lima bean leaves infested with

caterpillars of *Spodoptera exigua* or *Mythimna separata*. Methyl salicylate also was not recorded from maize and cowpea plants treated with regurgitant of the caterpillar *Spodoptera littoralis* (Fritzsche-Hoballah et al., 2002). This raises the question whether methyl salicylate emitted by different plant species is specifically induced by the spider mite *T. urticae*. However, the emission of methyl salicylate is also induced in pear leaves by psyllids (Scutareanu et al., 1997), in potato leaves by Colorado potato beetles (Bolter et al. 1997), and in *Arabidopsis thaliana* by *Pieris rapae* caterpillars (Van Poecke et al., 2001). Moreover, tobacco (*Nicotiana attenuata*) leaves infested with hornworm larvae (*Manduca quinquemaculata*) emitted a significantly larger amount of methyl salicylate than mechanically damaged leaves (Kessler and Baldwin, 2001). Shulaev et al. (1997) suggested that methyl salicylate, which is a volatile compound derived from salicylic acid, is a key compound in the induced resistance to fungal, bacterial, or viral pathogen attack (Metraux et al., 1990; Ryals et al., 1995; Malamy et al., 1996; Karban and Baldwin, 1997). Salicylic acid has also been reported to mediate whitefly- and aphid-induced plant responses (Walling, 2000; Moran and Thompson, 2001).

Nitrogen-Containing Compounds. In some plant species, nitrogen-containing compounds such as oximes and nitriles were induced by the mites. Oximes were found in the headspace of golden chain, black locust, and eggplant. The nitriles 2- and/or 3-methylbutanenitrile were found in the headspace of hop, black locust, golden chain, and eggplant. Both oximes and nitriles have been found in spider mite-infested lima bean, gerbera, cucumber, and jasmonic acid-treated lima bean plants (Takabayashi et al., 1994b; Dicke et al., 1999; Krips et al., 1999). Kaiser (1993) suggested that these nitrogen-containing volatile compounds were synthesized from amino acids.

(E,E)-α-Farnesene. Spider mite-infested golden chain leaves emitted the induced compound (*E*,*E*)-α-farnesene in large amounts. The compound was also induced in mite-infested soybean, black locust, tobacco, hop, grapevine, and ginkgo. (*E*,*E*)-α-Farnesene has been found in the headspace of *Psylla*-infested pear, together with methyl salicylate (Scutareanu et al., 1997). Both compounds attracted anthocorid predators in an olfactometer. Because (*E*,*E*)-α-farnesene is present in large amounts in the volatile blend of golden chain it might be a defected compound for the predatory mites. However, for golden chain, it remains to be investigated whether spider-mite infestation results in attraction of predatory mites and whether (*E*,*E*)-α-farnesene plays a role in that.

Qualitative vs. Quantitative Differences. In response to spider mite-infestation, each of the investigated plant species had its own volatile profile that comprised novel compounds (specific) but also compounds that were emitted in larger amounts compared to mechanically damaged leaves (nonspecific). Dicke et al. (1998) considers an herbivore-induced blend to show qualitative differences compared to a blend from mechanically damaged leaves when the novel

compounds are major blend contributors that are not produced in response to mechanical damage. The production of dominant novel compounds during spider mite-infestation was shown for all tested plant species, except eggplant and tobacco (both Solanaceae species). The dominant compounds in the blend of mite-infested eggplant leaves consisted of nonspecific compounds. Major compounds of the spider mite-induced blend of tobacco leaves resemble the major compounds of the blend emitted by clean leaves, but not those of mechanically damaged leaves, which is dominated by green leaf volatiles, i.e., (*Z*)-3-hexen-1-ol. Previous studies have shown that plant species of the Fabaceae (Dicke et al., 1990; Du et al., 1998) show qualitative differences in their volatile blends when infested by spider mites or aphids, while plant species of the Solanaceae show mainly quantitative differences (Bolter et al., 1997; Dicke et al., 1998). The present study confirms this.

Direct vs. Indirect Plant Defense. The question whether plant species use a combination of direct and indirect plant defense has not yet been clearly answered. Dicke et al. (1998) hypothesized that plant species that show a quantitative difference between volatile blends emitted from herbivore-damaged and mechanically damaged plants already have a high level of direct defense. Therefore, these species would reap no ecological benefit from inducing novel compounds for predators. To get an indication or the degree of direct defense of the investigated plant species, the degree to which the spider mite *T. urticae* accepted them has been investigated (Van Den Boom et al., 2003). To investigate the degree of direct defense, the migration rate of spider mites placed on a test plant leaf disc was measured over time. When the mites started wandering around, they could cross a plastic bridge and reach the other side of the bridge where they entered a lima bean leaf disc that functioned as a trap. The degree of indirect defense was indicated by the degree of predatory mite attraction towards plant odors of spider mite-infested plant species (Van Den Boom et al., 2002). Table 4 gives an overview of the direct and indirect defenses of all the investigated plant species.

Based on these results, it can be concluded that plant species with a low direct defense level use indirect defense to defend themselves, but do not always invest in the production of novel compounds. However, plant species with a high level of direct defense seem to invest in the production of novel compounds as well. For example, for ginkgo, it does not seem necessary to invest in specific compounds to strengthen the use of indirect defense because this plant already possesses a strong direct defense. However, ginkgo leaves showed the emission of novel compounds that dominated the blend after treatment with jasmonic acid. This suggests that the ability of plants to induce biosynthetic pathways that result in the emission of novel volatiles has originated early in the evolution of plants.

In summary, all the investigated plant species emitted one or more novel compounds when spider mite-infested leaves were compared to mechanically damaged leaves. This indicates that during spider-mite feeding on different plant species several biosynthetic pathways are induced. The induction of novel pathways and

TABLE 4. DIRECT VERSUS INDIRECT DEFENCE

Plant species	Direct defense[a]	Indirect defense[b]	Specificity of indirect defense[c]
Fabaceae			
Soybean	Very weak	Very strong	High
Golden chain	Very weak	—[d]	High
Black locust	Weak	—	High
Cowpea	Weak	Less strong	High
Solanaceae			
Tobacco SR1	Very weak	Less strong[e]	Low
Eggplant	Weak	Less strong	Low
Thorn apple	Less strong	—	High
Sweet pepper	Strong	Very strong	High
Other families			
Hop	Very weak	Less strong	High
Grapevine	Less strong	Very strong	High
Ginkgo	Very strong	Less strong	High

[a] Direct defense in percentages of *T. urticae* that had left a leaf disc of the indicated plant species within 15 min (Van Den Boom et al., 2002a): Very strong: 80–100%, Strong: 60–80%, Less strong: 40–60%, Weak: 20–40%, Very weak: 0–20%.
[b] Indirect defense in significant percentages when at least 40 predators of *P. persimilis* were tested on infested leaves vs. uninfested leaves in an olfactometer (Van Den Boom et al., 2002b). For all plant species the predatory mites were significantly attracted towards the infested leaves; "very strong" indicates that the percentage of productions towards infested leaves was 72–82%; "less strong" indicates that the percentage of preclators was lower than 67%.
[c] Specificity of indirect defense: High: presence of novel compounds that are dominant in the blend, Low: presence of novel compounds that are only present in small amounts.
[d] Not investigated.
[e] M. Dicke and H. Dijkman. (unpublished results).

compounds is a sophisticated way of indirect defense, because it is likely to increase the probability that the predatory mites discriminate herbivore-infested plants from mechanically damaged plants. It was hypothesized that plant species with a low degree of direct defense would invest in indirect defense by the induction of specific volatiles. Indeed, qualitative differences in spider mite-induced volatile blends are more prominent in the Fabaceae than in the Solanaceae. However, this tradeoff is not obvious for plant species with a weak direct defense level against spider mites, such as tobacco and eggplant. They do not invest in the production of novel compounds. For tobacco, however, the plant invests in direct defense by the production of nicotine in response to herbivore attack. This induced response has considerable costs in terms of fitness (Baldwin, 1999). For all investigated plant species, minor induced specific compounds or synergism among compounds might be responsible for predatory mite attraction. More research must be carried out to show which

compounds are important in the tritrophic interaction of each species, the spider mite, and the predatory mite *P. persimilis*.

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CHANGES IN CHEMICAL SIGNATURE AND HOST SPECIFICITY FROM LARVAL RETRIEVAL TO FULL SOCIAL INTEGRATION IN THE MYRMECOPHILOUS BUTTERFLY *Maculinea rebeli*

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Abstract—The ant social parasite, *Maculinea rebeli* shows high levels of host specificity at a regional scale. While 68–88% of caterpillars in the field are adopted by nonhost *Myrmica* ants, 95–100% of the butterflies emerge from the natural host *M. schencki* the following year. While retrieval of preadoption caterpillars is specific to the genus *Myrmica*, it does not explain differential survival with different *Myrmica* species. We present survival data with host and nonhost *Myrmica* species suggesting that, with nonhosts (*M. sabuleti* and *M. rubra*), survival depends on the physiological state of the colony. We also compared the similarities of the epicuticular surface hydrocarbon signatures of caterpillars that were reared by host and nonhost *Myrmica* for 3 weeks with those from tending workers. Counterintuitively, the hydrocarbons of postadoption caterpillars were more similar (78%, 73%) to the ant colony profiles of the nonhost species than were caterpillars reared in colonies of *M. schencki* (42% similarity). However, caterpillars from *M. schencki* nests that were then isolated for 4 additional days showed unchanged chemical profiles, whereas the similarities of those from nonhost colonies fell to 52 and 56%, respectively. Six compounds, presumably newly synthesized, were detected on the isolated caterpillars that could not have been acquired from *M. sabuleti* and *M. rubra* (nor occurred on preadoption caterpillars), five of which were found on the natural host *M. schencki*. These new compounds may relate to the high rank the caterpillars attain within the hierarchy of *M. schencki* societies. The same compounds would

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identify the caterpillars as intruders in non-*schencki* colonies, where their synthesis appeared to be largely suppressed. The ability to synthesize or suppress additional compounds once adopted explains the pattern of mortalities found among fully integrated caterpillars in *Myrmica* colonies of different species and physiological states.

Key Words—Host specificity, chemical mimicry, cuticular hydrocarbons, *Maculinea* butterflies, *Myrmica* ants, myrmecophily.

INTRODUCTION

Perhaps 80,000–100,000 insect species possess myrmecophilous adaptations enabling them to interact and coexist with ants (Hölldobler and Wilson, 1990; Elmes, 1996). Most associations are mutualistic and, with notable exceptions, involve relatively unspecialized adaptations that function with a range of ant partners (Pierce et al., 1987, 2002; Fiedler, 1991; DeVries et al., 1993). In contrast, perhaps 10–20% of myrmecophiles penetrate ant societies to exploit the fiercely protected resources inside ant nests (Schönrogge et al., 2002). This trait of social parasitism has evolved independently in at least 8 insect orders and perhaps 14 times within the butterfly family Lycaenidae (Hölldobler and Wilson, 1990; Fiedler, 1998; Pierce et al., 2002): it typically involves extreme specialization resulting in high levels of host-ant specificity (Thomas and Elmes, 1998; Elmes et al., 1999).

The adaptations that enable social parasites to infiltrate ant societies vary greatly among species according to the type of interaction, which ranges from evasion to full social integration (Hölldobler and Wilson, 1990). In theory, full integration with ants results in higher survival and a more efficient exploitation of the resource; the cost is increased host specificity, which may constrain some social parasites to exploiting local races of a single host species (Thomas and Elmes, 1998; Elmes et al., 1999). This dichotomy is illustrated within the lycaenid genus *Maculinea*, in which the larvae (hereafter called caterpillars) of two species (*M. arion* (L.), *M. teleius* Bergsträsser) are evasive predators of ant larvae, each adapted to one *Myrmica* species with which survival is four to five times higher than when inhabiting "nonhost" *Myrmica* colonies (Thomas et al., 1989; Thomas and Wardlaw, 1992). In contrast, *Maculinea alcon* Denis and Schiff and *M. rebeli* Hirschke are cuckoo-feeders, which mimic ant larvae and are fed directly, often by trophallaxis by nurse ants in the brood chambers (Elmes et al., 1991a). Typical populations of the cuckoo *Maculinea* species are highly specific to a single host species, apparently at the regional scale, since both butterflies switch hosts across their European ranges (Steiner et al., 2003); only near the boundaries of switches have populations been found displaying a "generalism" comparable to that of predatory species (Als et al., 2001, 2002).

Maculinea butterflies are atypical among social parasites in that their larvae feed on a specific foodplant before falling off and being adopted at the start of the

final instar by *Myrmica* ants. Nevertheless, myrmecophily dominates their lives, with caterpillars and pupae living 11–23 months inside *Myrmica* nests where they obtain >98% of their ultimate biomass (Elmes et al., 2001). Colony penetration is frequently cited as the key period in the life of a social parasite (Hölldobler and Wilson, 1990), and for the highly integrated cuckoo species, *M. rebeli*, we distinguished three phases: "retrieval" of caterpillars by worker ants from beneath the foodplant into ant nests; "initial integration" with the ant colony over the first 48 hr after retrieval, during which some individuals are quickly accepted but others are killed or neglected; and "full integration" with the ant's society, lasting until pupation 10–22 months later (Elmes et al., 2004).

The retrieval of *Maculinea* from beneath food plants has been studied in some detail using populations in France and Spain. In both types of species, the behavior of caterpillars greatly enhances their probability of being found by *Myrmica* workers rather than by ants of other genera, but wild caterpillars fail to bias their retrieval toward their *Myrmica* host species (Fiedler, 1990; Elmes et al., 1991b; Als et al., 2001; Thomas, 2002). Consequently, 68–88% of *M. rebeli* caterpillars were retrieved into colonies of nonhost *Myrmica* species in populations studied in the Hautes-Alpes and Pyrenees, yet 95–100% of adults emerged from *M. schencki* Emery nests the following year (Elmes et al., 1991a; Thomas and Elmes, 1998). The cue inducing retrieval is a cocktail of cuticular hydrocarbons that, when applied to glass dummies, triggers similar retrieval behaviors to dummies coated with extracts from *M. schencki* larvae (Akino et al., 1999). These hydrocarbon signatures of preadoption *M. rebeli* caterpillars were simpler (fewer compounds) than those detected on the five *Myrmica* species tested (Akino et al., 1999; Elmes et al., 2002) and induced retrieval by all *Myrmica* species, in the same way that any worker will retrieve the brood of a congener that is artificially placed in its territory. Nevertheless, the cuticular hydrocarbons of preadoption *M. rebeli* caterpillars mimic those of its host, *M. schencki*, more closely than other *Myrmica* species (Akino et al., 1999; Elmes et al., 2002).

Host specificity of *M. rebeli* caterpillars during the "initial integration" phase had not been quantified, but our anecdotal observations suggested higher survival with *M. schencki* than with other *Myrmica* species; we hypothesized that this resulted from the closer match of the hydrocarbon signature on retrieved caterpillars when first placed in *M. schencki* brood chambers (Elmes et al., 2002). In addition, Als et al. (2001) demonstrated that *M. alcon* caterpillars are retrieved more often from the outer chamber of laboratory "nests" by their host *Myrmica* species, probably referring more to the initial integration process than to the definition of retrieval from food plants used here.

Although *M. schencki* workers attend dummies dosed with hydrocarbon extracts from their kin and nonkin larvae (in that order) more frequently than those containing the simpler preadoption *M. rebeli* extracts (Akino et al., 1999), the social status of caterpillars quickly rises in their nests, and within a week they are

preferred even to large kin larvae (Thomas et al., 1998). By now, their hydrocarbon signature is more complex and closely resembles that of their host. We suggested that this was an example of "chemical camouflage" (*sensu* Dettner and Liepert, 1994) rather than chemical mimicry, and resulted simply from the missing chemicals in *M. rebeli*'s preadoption profile being passively acquired through intimate contact with the ants (Akino et al., 1999).

This "chemical camouflage" hypothesis now seems naive. It fails to explain why caterpillars outcompete ant larvae of a similar size for worker attention when *M. schencki* nests are perturbed (Thomas et al., 1998), or why survival ranges from low to zero in wild (but not laboratory) colonies of nonhost *Myrmica* species, whose species or colony *gestalt* should be acquired with a similar inevitability (Elmes et al., 2002). Recent experiments shed some light on this discrepancy in mortalities (Elmes et al., in press). Survival is high with any *Myrmica* species when food is abundant, but if demand exceeds supply (a common occurrence in the field), *M. rebeli* caterpillars are abandoned or eaten by nonhost *Myrmica* species, whereas *M. schencki* workers continue to tend and feed caterpillars at their own expense. We suggested a new hypothesis to explain this. To achieve full integration and a high enough status to outcompete ant larvae in the host's societies, older *M. rebeli* caterpillars perhaps secrete additional chemicals that mimic *M. schencki*'s full signature more closely than those found on the preadoption caterpillars (Elmes et al., 2002; Elmes et al., 2004).

Here we collate the disparate evidence that the host specificity of *M. rebeli* caterpillars changes in intensity between retrieval from food plants to full social integration with *M. schencki* societies, and, for the first time, quantify variation in host specificity during the "initial integration" phase. We then test the hypothesis that caterpillars supplement any camouflaging chemicals acquired from *M. schencki* inside the nest by secreting additional mimetic chemicals.To study this, we analyzed the cuticular hydrocarbon profiles of *M. schencki* and two nonhost species of *Myrmica*, and compared them with analyses of the profiles of preadoption caterpillars and older caterpillars that had lived 3 weeks with each ant species. Finally, we analyzed the chemicals on 3.5-week-old final instars that had been isolated from their ant colonies for 4 days, which approximates the time taken by other social parasites to lose most acquired chemicals (Dettner and Liepert, 1994).

METHODS AND MATERIALS

Changing Phases of Host Specificity. Published measurements of host specificity in *M. rebeli* caterpillars from the Hautes-Alpes (France) and Pyrenees (Spain) during the first (retrieval to nests) and final phases (full integration) of host colony penetration, involving both well-fed and impoverished *Myrmica* colonies, were combined with analyses of unpublished records of survival during the first 48 hr

("initial integration") of 258 freshly molted final instars that were introduced into laboratory cultures of *Myrmica schencki* (natural host) (12 nests), *M. sabuleti* Meinert (9 nests), and *M. rubra* L. (9 nests) (nonhosts). Experimental procedures are described by Elmes et al. (1991a) and Wardlaw et al. (2000), Analyses were restricted to cultures where density effects in autumn were negligible (>10 workers per caterpillar, Thomas et al., 1993) and where survival with all three *Myrmica* species was measured in the same experiment.

Materials. *M. schencki* colonies were collected in the Alps and colonies of *M. rubra* and *M. sabuleti* from SW England. The foodplants, *Gentiana cruciata* L., with eggs of *Maculinea rebeli*, were collected in the Hautes Alpes, where *Myrmica schencki* is their principal host species (Thomas et al., 1989). Larvae on food plants were collected using a protocol that ensures no damage to the natural populations and reared until their final instar in the laboratory.

Experimental Design. Preadoption caterpillars were sampled within 6 hr of leaving their foodplant and never had contact with ants. Four samples of surface hydrocarbons were taken using 10 caterpillars per sample. Caterpillars were transferred into a clean glass vial and extracted by submerging them under 200-μl hexane for 20 min. The hexane was decanted and sealed into a clean glass ampoule under N_2 until analysis. Hereafter, the term "extraction" refers to the above procedure and "extract" to a sample of cuticular hydrocarbons thus obtained. The cuticular hydrocarbons of five workers from each ant colony were also extracted.

To obtain extracts from fully integrated individuals, three, 4th instars were introduced into laboratory nests (150 workers) of the natural host *M. schencki* (7 nests) and of the nonhosts *M. sabuleti* (5 nests) and *M. rubra* (5 nests). Caterpillars that died or failed to integrate at this initial phase were replaced so that each nest reared three caterpillars. The ants were fed an ample diet of sugar and *Drosophila* larvae throughout the experiment (Wardlaw et al., 1998). After 3 weeks, the largest caterpillar from each nest was extracted, while the others were isolated from their ants and each other at 8°C and without food for another 4 days before the larger caterpillar from each replicate was also extracted. We extracted only from the fast-developing morph of *M. rebeli* caterpillars (Schönrogge et al., 2000) to avoid the possibility that slow-developing (2-year) individuals contain fewer or different chemicals.

Chemical Analysis. All extracts of *M. rebeli* caterpillars were concentrated to 20 μl and those of ant workers to 50 μl. Two microliters of every sample, equivalent to 10% of a caterpillar and 20% of a worker ant, were analyzed by gas chromatography with mass spectrometric detection (GC–MSD) using an HP 5890II GC and HP 5971A MSD. The GC was equipped with an HP1 capillary column of dimensions: 50 m $\times$ 0.32 mm i.d. with a 0.52-μm phase-coating thickness. The carrier gas was ultrahigh purity helium with a column head pressure of 10 psi. The oven was programmed to ramp from 35 to 300°C at 10°C/min and then held for 40 min. The transfer line was maintained at 280°C. Splitless injection (1-min

hold) was performed using an automated sampler with the injector maintained at 250°C. MS data were acquired in full-scan mode over a range of 40–600 *m/z*. Mass chromatograms were initially screened for hydrocarbons by examining the selected ion chromatogram of $m/z = 57$. The chromatogram was integrated at a threshold value of 12 (HP integrator) to determine the total ion count. With each sequence of samples, we also analyzed alkane standards (*n*-C20–*n*-C36). The position of each peak within that range in a sample was calculated as an Equivalent Chain Length (ECL) according to Elmes et al. (2002). Mass chromatograms were visually inspected to ensure that they were free of gross interferences and that all peaks of interest were alkanes or alkenes, and that peaks were chromatographically well resolved and symmetrical. Peaks of interest were tentatively identified by using a combination of ECL number, inspection of their full scan mass spectra, and matching with the NIST-97 mass spectral database.

Statistics. Within each sample, the area under each peak (ECL number) was expressed as the proportion of the sum over the area of all peaks in the chromatogram. Samples were compared using multivariate and nonparametric multidimensional scaling (MDS) on the ranks of the Bray–Curtis similarities (Carr, 1996). The extent of a final lack of fit was assessed by a STRESS statistic (STandardised REsidual Sum of Squares), a method widely used in pairwise comparisons of species compositions in ecological assessments. Elmes et al. (2002) describe its application to analyses of cuticular hydrocarbon mixtures and discuss its sensitivity. Pairwise differences between species and treatments were assessed using an analysis of similarities (ANOSIM) (Clarke, 1993). All statistics were carried out with PRIMER 5 (PRIMER-E, Plymouth, UK).

RESULTS

Host Specificity

Retrieval and Survival. Survival of *M. rebeli* caterpillars during their initial integration period was significantly different among the three *Myrmica* ants (analysis of deviance, general linear model, binomial errors corrected for overdispersion, on deletion $F_{(2,27)} = 10.23$, $P < 0.001$). The proportion surviving being higher ($F_{(1,28)} = 19.65$, $P < 0.001$) in *M. schencki* (host) colonies (mean proportion 0.90) than either *M. sabuleti* (mean proportion 0.50) or *M. rubra* (mean proportion 0.58) with no significant difference in survival rates with the two nonhost ants. This completes the sequence of changing host specificities from the caterpillar's final mult to its full (or maximum) integration within a *Myrmica* society for the *schencki*-using race of *M. rebeli* (Table 1). Caterpillars were retrieved by the first *Myrmica* worker to encounter them in the field, regardless of species; however, survival was up to twice as high inside colonies of the host ant during the first 48 hr of initial integration; for any individual surviving that had a near 100%

TABLE 1. HOST SPECIFICITY IN *Maculinea rebeli* CATERPILLARS WITH "HOST" (*M. schencki*) AND "NONHOST" (*M. sabuleti*, *M. rubra*) ANTS IN THE THREE PHASES DURING WHICH THEY INFILTRATE *Myrmica* SOCIAL SYSTEMS

Ant	% retrieval: plant to ant colony (first evening)[a]	% survival: initial integration with ant colony (day 0–2)[b]	% survival with ants after day 2 food > demand[c]	% survival with ants after day 2 food < demand[c]
M. schencki	100	89	99	88
M. sabuleti	100	59	100	13
M. rubra	Not tested	45	100	33

[a] Retrieval; undisturbed wild caterpillars, $N = 42$ for each ant, *M. rubra* not tested (from Elmes et al., 1991b).

[b] Initial integration: new data, $N = 12, 9, 9$ colonies, respectively of *M. schencki*, *M. sabuleti*, and *M. rubra*, $P < 0.05$ $\text{survival}_{\text{schencki}} > \text{survival}_{sabuleti/rubra}$ (general linear model, binomial errors $F_{1,28} = 19.68$, $P < 0.001$).

[c] Maximally integrated caterpillars with surplus (*ns*) or insufficient food ($P = 0.001$ $\text{survival}_{\text{schencki}} > \text{survival}_{\text{sabuleti/rubra}}$) (from Elmes et al., 2004).

chance of survival through the autumn feeding period, so long as food remained plentiful, but during periods of food shortage, survival was 2.5–6.8 times greater in *M. schencki* colonies compared to nonhost ants (Table 1). The net effect, under the conditions of our resource-limited experiment, is that final instars on gentians growing at low densities in the territories of host *Myrmica* colonies have a 78% chance of surviving their first period of ant exploitation (to hibernation), whereas those in the territories of nonhost species have a 8–15% chance of survival.

Caterpillar Weights in the Chemical Extraction Experiments. The mean weights of *M. rebeli* caterpillars reared for 3 weeks by *M. schencki* (12.6 mg $\pm$ 1 mg), *M. sabuleti* (11.2 mg $\pm$ 0.7 mg), and *M. rubra* (10.6 $\pm$ 1.1 mg) showed no significant differences. After 4 days of isolation and without food, the weights in all three groups had decreased (caterpillars from *M. schencki* nests: 9.6 $\pm$ 1.1 mg; *M. sabuleti* nests: 8.1 $\pm$ 1 mg; *M. rubra* nests: 6.4 $\pm$ 0.6 mg), with caterpillars from *M. rubra* nests being significantly lighter than those from *M. schencki* nests ($t = 2.66$, $df = 9$, $P < 0.05$).

Bray–Curtis Similarities of Chemical Profiles. We confirmed the result of Akino et al. (1999) that the hydrocarbon profile of preadoption *M. rebeli* caterpillars was closer to that of their host *M. schencki* (6.9%) than it was to the two nonhost species *M. sabuleti* (2.3%) and *M. rubra* (4.0%; Figure 1, Table 2), and that all these similarity indexes were low because of the small number of chemicals secreted by preadoption caterpillars; in our experiment, nine hydrocarbons were identified initially on *M. rebeli*, of which 7, 2, and 3.5, respectively, matched the 34, 18, and 22 hydrocarbons found on *M. schencki*, *M. sabuleti*, and *M. rubra*.

The chemical similarity to ants of caterpillars kept inside *M. schencki* nests for 3 weeks increased to 43.0%. After 4 further days of isolation from ants, the

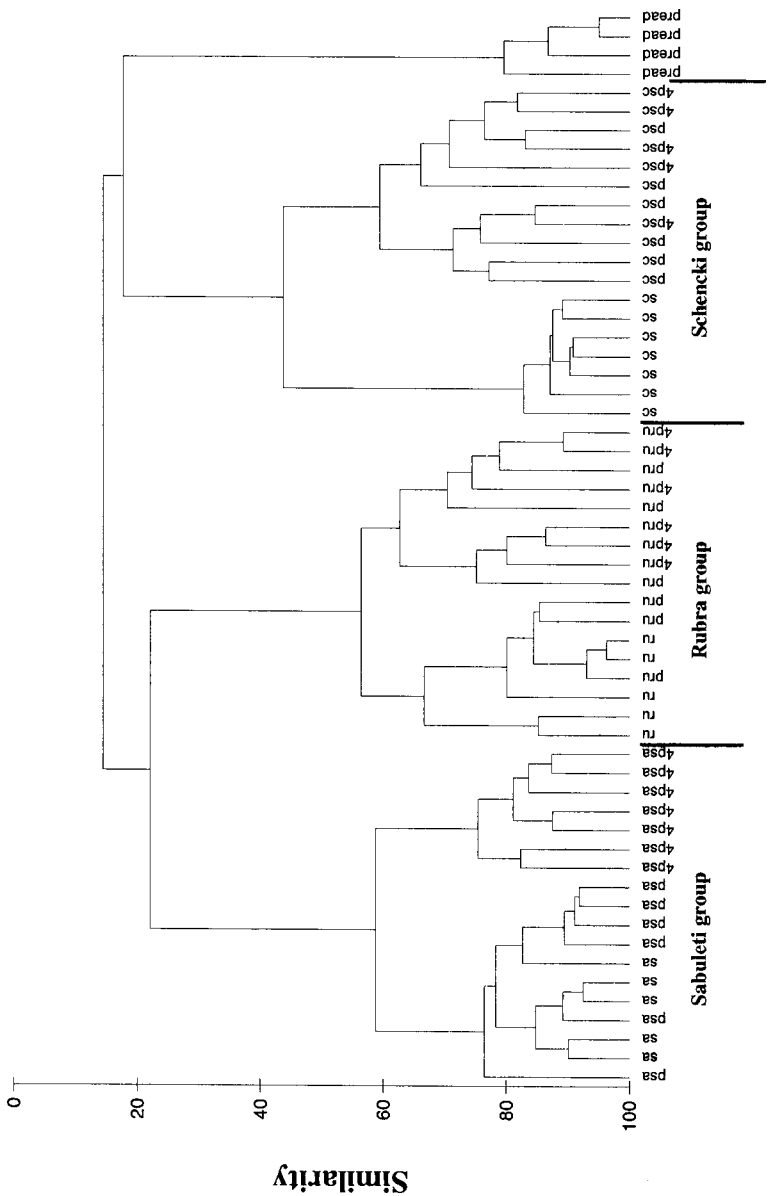

FIG. 1. Dendrogram of similarities of hydrocarbon signatures between the three ant species *Myrmica schencki* (sc), *M. sabuleti* (sa), and *M. rubra* (ru); caterpillars that lived for 3 weeks in the colonies of the 3 species (psc, psa, and pru); caterpillars living for 3 weeks in the colonies after being kept in isolation for an extra 4 days (4psc, 4psa, and 4pru); and preadoption caterpillars that had never been exposed to ants.

TABLE 2. MATRIX COMPARING THE SIGNIFICANCE LEVELS OF GROUP ANALYSIS OF SIMILARITIES (UPPER HALF) AND MEANS BRAY–CURTIS SIMILARITIES (UNTRANSFORMED ± SE; LOWER HALF) OF *Maculinea rebeli* CATERPILLARS REARED FOR 3 WEEKS IN THE NESTS OF 3 *Myrmica* SPECIES

	Preadoption caterpillar	*M. schencki* worker	Postadoption (sch) 3 weeks	Postadoption (sch) 4 days	*M. sabuleti* worker	Postadoption (sab) 3 weeks	Postadoption (sab) 4 days	*M. rubra* worker	Postadoption (rub) 3 weeks	Postadoption (rub) 4 days
Preadoption caterpillar	84.5±2.8 (6)[b]	**[a]	**	**	**	**	**	**	**	**
M. *schencki* worker	6.9 ± 0.4 (28)	85.6 ± 0.7 (21)	**	**	**	**	**	**	**	**
Postadoption (sch) 3 weeks	19.6 ± 2.0 (24)	43.0 ± 1.8 (42)	61.7 ± 3.4 (15)	ns	**	**	**	**	**	**
Postadoption (sch) 4 days	30.6 ± 1.7 (20)	44.7 ± 1.4 (35)	65.6 ± 1.7 (30)	71.5 ± 1.8 (10)	**	**	**	**	**	**
M. sabuleti worker	2.3 ± 0.2 (20)	1.0 ± 0.1 (35)	8.4 ± 0.8 (30)	6.4 ± 0.3 (25)	84.2 ± 2.1 (10)	*	**	**	**	**
Postadoption (sab) 3 weeks	5.7 ± 0.6 (20)	2.1 ± 0.1 (42)	14.6 ± 1.0 (36)	13.1 ± 0.7 (30)	78.3 ± 1.1 (30)	84.9 ± 1.5 (15)	**	**	**	**
Postadoption (sab) 4 days	19.5 ± 1.0 (28)	6.6 ± 0.3 (49)	30.5 ± 1.9 (42)	33.6 ± 1.1 (35)	52.6 ± 1.4 (35)	63.9 ± 1.4 (42)	79.3 ± 1.1 (20)	**	**	**
M. rubra worker	4.0 ± 0.4 (20)	8.2 ± 0.3 (35)	15.1 ± 0.8 (30)	13.8 ± 0.7 (25)	12.4 ± 0.7 (25)	14.4 ± 0.8 (30)	16.0 ± 0.8 (35)	77.2 ± 3.3 (10)	**	**
Postadoption (rub) 3 weeks	6.0 ± 1.0 (24)	10.4 ± 0.3 (42)	25.3 ± 1.0 (36)	22.0 ± 1.0 (30)	15.7 ± 0.5 (30)	20.4 ± 0.7 (36)	25.4 ± 1.1 (42)	66.8 ± 2.5 (30)	73.7 ± 1.8 (15)	**
Postadoption (rub) 4 days	13.0 ± 1.8 (24)	10.5 ± 0.4 (42)	33.1 ± 1.2 (36)	29.6 ± 1.6 (30)	20.9 ± 0.8 (30)	26.4 ± 0.9 (36)	37.9 ± 1.4 (36)	56.6 ± 2.2 (45)	63.3 ± 2.2 (41)	69.3 ± 3.1 (15)

Note. The lower half of the matrix shows the mean Bray-Curtis similarities (untransformed ± SE) between the chemical profiles of the preadoption caterpillars of *M. rebeli*, the workers of *M. schencki*, *M. sabuleti*, and *M. rubra*, *M. rebeli* caterpillars after remaining for 3 weeks in the nests of *M. schencki* (sch), *M. sabuleti* (sab), and *M. rubra* (rub), and *M. rebeli* caterpillars that were isolated for 4 days after 3 weeks in the nests of the three *Myrmica* species. The upper half shows the significance levels of a between groups analysis of similarities.

[a] (ANOSIM-* $P < 0.05$; ** $P < 0.01$; $P > 0.05$ (NS).

[b] N = Number of pairwise similarities.

index was 44.7% (no significant difference). If all 22 additional compounds on caterpillars that increased the similarity to the host ants from the preadoption to the postadoption state were acquired, we would expect the isolated caterpillars to lose some of them, resulting in a decreased similarity.

M. rebeli caterpillars kept with *M. sabuleti* and *M. rubra* showed a different chemical pattern. *M. sabuleti* workers differed the most from the natural host *M. schencki*, with only 1.05% similarity in profiles compared to *M. rubra*'s 8.2% match (Table 2). The similarly of caterpillars that lived for 3 weeks in *M. sabuleti* colonies increased twofold (78.3%) compared to caterpillars living in *M. schencki* nests (43.0%, Table 2). Moreover, in contrast to the caterpillars from *M. schencki* colonies, those reared with *M. sabuleti* nests became less similar, (52.6%) to that ant after being isolated from it for 4 days (Table 2). During this period, they became slightly—but significantly—more similar to the natural host *M. schencki* (6.6% compared to 2.1% when first removed from the *M. sabuleti* nests). The trend in similarity between ants and caterpillars reared by *M. rubra* followed the same pattern as with *M. sabuleti*. After 3 weeks in *M. rubra* nests, caterpillars were more similar (66.8%) to this unnatural host than those in *M. schencki* nests were to their natural host. After 4-days isolation, the similarity of the former to *M. rubra* dropped to 56.6%. However, there was no significant change in the similarity of these caterpillars toward *M. schencki*'s hydrocarbon profile (10.4% after 3 weeks and 10.5% after the additional 4 days, Table 2).

These patterns are illustrated by a hierarchical agglomerative cluster analysis on the group averages of the similarities (Figure 1) and the nonparametric MDS plot (Figure 2a). While the preadoption caterpillars (pread) are well separated from their host *M. schencki* (sc), they are closer to it than to the other *Myrmica* species. Within the "*schencki* treatment," the 3-week caterpillars (psc) and those that were isolated for 4 days after 3 weeks with the ants (4 psc) form one group that is distinctly separate from *M. schencki*. In contrast, within the "*sabuleti* treatment," the 3-week caterpillars (psa) form one group very close to the ants (sa) that is well separated from the caterpillars that were isolated for 4 days (4 psa). Finally, within the "*rubra* treatment," the ants (ru) and the 4-day isolated caterpillars (4 pru) form separate groups, but the caterpillars that were extracted after 3-weeks with the ants span both groups.

Relative Abundance vs. Presence/absence. Little is known about the importance as a signal of any individual compound within the hydrocarbon signature of each *Myrmica* species. Nor is it known if recognition depends on the relative amounts or the presence/absence of individual compounds. We calculated Bray–Curtis similarities on untransformed data and on square root, fourth root, and presence/absence transformed data, which has the effect of transferring the emphasis from relative amounts to presence/absence of compounds. Figure 2 shows the relative positions of each sample on MDS plots following the transformations. The relative positions of the clusters are not affected when either relative abundance or

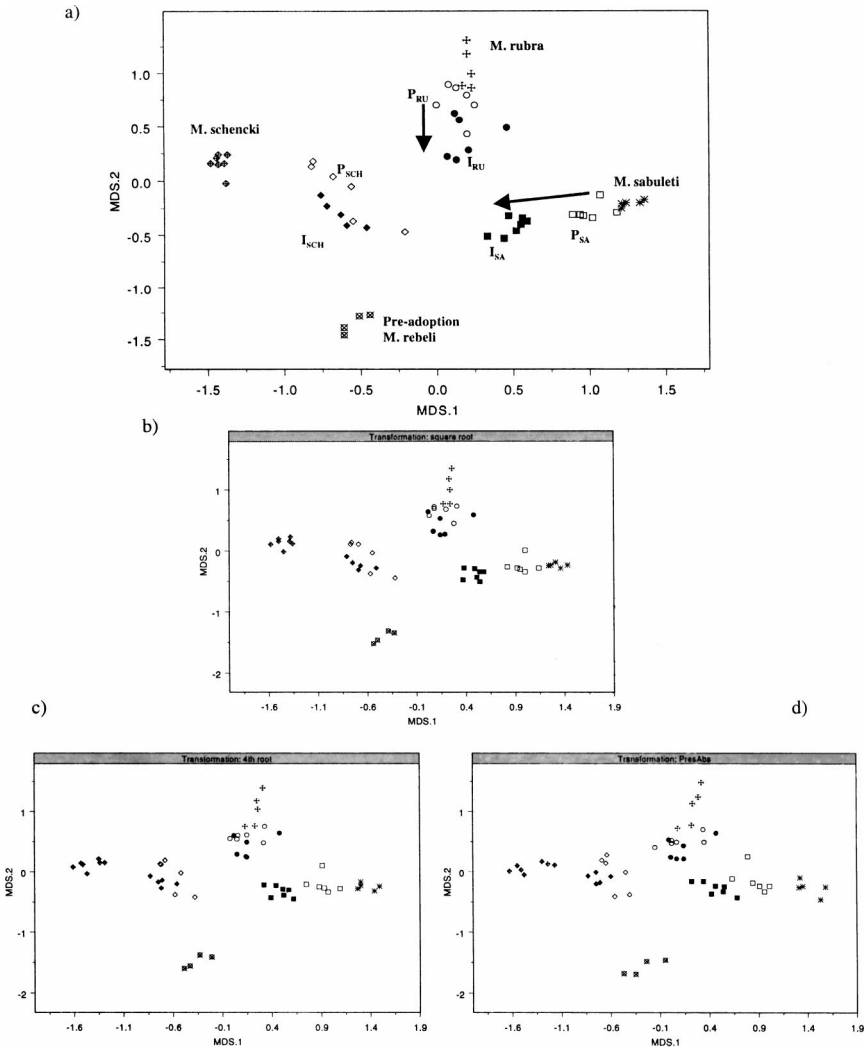

FIG. 2. MDS plots of Bray–Curtis similarities of hydrocarbon signatures: (a) not transformed, (b) after square root transformation, (c) after fourth root transformation, and (d) presence/absence similarities. The different groups are indicated in Figure 2a. $P_{subscript}$ indicates samples from *M. rebeli* caterpillars after 3 weeks in the host ant nest, while $I_{subscript}$ indicates caterpillars after 4-days isolation. The subscripts refer to the *Myrmica* species that reared the caterpillars: SA – *sabuleti*, SCH – *schencki*, and RU – *rubra*.

presence/absence is emphasized, although most clusters are tighter when the data are untransformed. The STRESS values for the four plots (as measures of fit) vary little, but increase from 0.08 for untransformed and square root transformed data to 0.09 for the fourth root transformation to 0.11 for presence/absence data. Carrying out an ANOSIM under each transformation shows only one change in the significant pairwise differences (Table 2) for untransformed data: with untransformed or square root transformed data, the hydrocarbon signatures of *M. rebeli* caterpillars after 3 weeks in *M. rubra* nests differ significantly (ANOSIM $P < 0.01$ and $P < 0.05$) from those isolated for 4 days from ants. Using fourth root or presence/absence data, which take little or no account of the relative concentrations of compounds, the difference is not significant.

Postadoption Synthesis or Acquired Hydrocarbons. Caterpillars that lived for 3 weeks in *M. sabuleti* colonies and then in isolation for 4 days had six surface compounds that were found in all (or all but one) of their extracts (Table 3). These compounds had ECL values of 24.0, 26.6, 29.3, 30.0, 31.0, and 31.3, and were tentatively identified as straight chain or methyl-branched alkanes. They were assigned the following structures on the basis of a combination of ECL value comparisons, mass spectral database matching, and mass spectral interpretation: 24.0: *n*-C24; 26.6: 4-/2-methyl-C26; 29.3: 11-/13-/15-methyl-C29; 30.0: *n*-C30; 31.0: *n*-C31; 31.3: 11-/13-/15-methyl-C31.

These six compounds are of particular interest because they fall into at least one of three categories:

(1) Compounds *n*-C30 and *n*-C31 were present on the preadoption caterpillars and those after 3 weeks in *schencki* nests, but on only 33% of the caterpillars reared with *M. sabuleti*. Furthermore, *n*-C30 and *n*-C31 were not detected in any *M. sabuleti* or *M. rubra* worker sample. They could not have been acquired from the ants, but must have resulted from continued synthesis by the caterpillars after the adoption.

(2) Compounds 11-/13-/15-methyl-C29 and 11-/13-/15-methyl-C31 were detected on neither the preadoption caterpillars nor the *M. sabuleti* workers, but were found consistently on the postadoption caterpillars and on all caterpillars that were isolated from the ants for 4 days. These compounds appear to be synthesized, but only after the caterpillar has contact with the *Myrmica* ants; they make caterpillars more "*schencki*-like" compared to their preadoption state. That 11-/13-/15-methyl-C29 was only detected on half the caterpillars that were reared by *M. sabuleti*, and 11-/13-/15-methyl-C31 on none and on only 67% of those reared by *M. rubra*, suggests that the synthesis of these compounds can be suppressed when caterpillars are in nonhost colonies.

(3) Compound *n*-C24 was not detected on workers of the host *M. schencki* or on preadoption caterpillars, but was present in all samples of postadoption

TABLE 3. FREQUENCY OF DETECTION (%) OF POSTADOPTION HYDROCARBONS ON *M. rebeli* CATERPILLARS UNDER DIFFERENT TREATMENTS, ON PREADOPTION CATERPILLARS AND ON HOST AND NONHOST WORKERS

Compounds equivalent chain length	*Myrmica* workers			*Maculinea rebeli*						
	M. schencki	*M. rubra*	*M. sabuleti*	Preadoption	Schencki (+3 weeks)	Rubra (+3 weeks)	Sabuleti (+3 weeks)	Schencki (+4 days)	Rubra (+4 days)	Sabuleti (+4 days)
24.0	0	40	100	0	100	100	100	100	100	100
26.6	43	100	0	25	100	100	100	100	100	100
29.3	100	100	0	0	100	100	50	100	100	86
30.0	100	0	0	100	100	83	33	100	100	100
31.0	100	0	0	100	100	67	33	100	83	100
31.3	100	40	0	0	100	67	0	100	83	100

Note. M. rebeli caterpillars were extracted after 3 weeks (+3 weeks) in nests of *M. schencki* (host), *M. rubra*, and *M. sabuleti*. Caterpillars from the same nests and after 3 weeks rearing were also isolated for 4 days (+4 days) before extraction.

caterpillars. Thus, $n-$C24 indicates that not all *de novo* postadoption synthesis is aimed to make the caterpillars more like *M. schencki* workers or larvae.

DISCUSSION

Our results confirm that two phases of host specificity exist during the integration of *M. rebeli* caterpillars with *Myrmica* societies. They also complete the sequence of changing ant specificity between oviposition and pupation in this species. There is an initial ca. 48-hr period once inside a *Myrmica* colony when nearly twice as many individuals are accepted by societies of the natural host, and a later, more distinct differentiation between the surviving caterpillars with different *Myrmica* species once the ants' food supply falls below demand (Table 1).

We confirmed that the hydrocarbon signatures of the host *M. schencki* and the preadoption caterpillars of *M. rebeli* are more similar to each other than *M. schencki*'s is to any of the other *Myrmica* species (Akino et al., 1999; Figure 1), despite using ants and caterpillars collected from different massifs and a different technique to analyze similarities. Little is known about the function of individual or classes of compounds within the mixtures of cuticular hydrocarbons or how they work (presence/absence vs. relative amounts) in any social insect system. Compared to *Myrmica*, the preadoption *M. rebeli* caterpillars have few (12) cuticular hydrocarbons, of which 7 are straight chain alkanes and 5 are methylated alkanes (Akino et al., 1999). None of the methylated alkanes makes up more than 5% of the total amount of alkanes. Elmes et al. (2002) suggested that the dominance of straight chain alkanes renders the caterpillar "chemically insignificant" and, thus, escapes ant it attack or attention (Lenoir et al., 2001). However, a positive interaction is evident during the retrieval of caterpillars by workers from beneath food plants. Either this is solely based on the five-methylated alkanes, or straight chain alkanes are informative in this system as observed elsewhere (Vander Meer and Morel, 1998; Viana et al., 2001; Elmes et al., 2002). Further behavioral experiments are needed to decipher these chemical codes (Dani et al., 2001).

Once inside a nest, caterpillars were originally thought to acquire their ant colony's odor, enabling them to interact with the ants. However, if so, caterpillars should be able to survive in colonies of any *Myrmica* species. Two compounds, *n*-tetracosane and 4-methyltetracosane, were previously detected on postadoption caterpillars in *M. schencki* nests that could not have originated from the *M. schencki* worker ants, and were absent from preadoption caterpillars, suggesting that the caterpillars synthesize new compounds after they come in contact with the ants (Elmes et al., 2002). By introducing *M. rebeli* caterpillars to colonies of *M. sabuleti*—the species which occupies the niche closest to *M. schencki* and adopts most *M. rebeli* caterpillars in the field, but which is chemically the most dissimilar known *Myrmica* species to *M. schencki*— we hoped to clarify which compounds

might be synthesized after adoption. We found that caterpillars living in *M. sabuleti* colonies for 3 weeks were more similar to these nonhost ants than others were to *M. schencki* after 3 weeks in their nests. We detected few compounds on these caterpillars that could not have originated from *M. sabuleti*, and those only on one or two individuals. In contrast, after 4 days of isolation from ants, we detected five new compounds on all extracted caterpillars that they could not have acquired from *M. sabuleti* workers; all matched compounds found on *M. schencki* workers.

This suggests that the synthesis of new cuticular compounds by the caterpillars may be suppressed in the presence of the nonhost *M. sabuleti*, and that the compounds found after 3 weeks were indeed acquired. The high similarity found between the 3-week caterpillars and *M. sabuleti* or *M. rubra* declined over the 4-day period of isolation, probably because of the loss of acquired compounds and the synthesis of new nonmatching ones. We predict that this trend would have continued; isolation for 4 days at 8°C represented a compromise between allowing some time for acquired compounds to dissipate while ensuring that the caterpillars—that were inevitably isolated from their food supply—remained fit. In contrast, we found no difference between the chemical composition on caterpillars kept for 3 weeks in *M. schencki* nests and those that were isolated for 4 days.

These results are consistent with experiments that showed that *M. sabuleti* and *M. rubra* that were semistarved, actively killed *M. rebeli* caterpillars, while *M. schencki* colonies treated the same way tended caterpillars at the workers' expense (70% survival after 3 weeks, Elmes et al., 2004). We suspect a double jeopardy may occur in the first situation. By relying on a (presumably weak) dose of acquired chemicals, not only are *M. rebeli* caterpillars low in the hierarchy in nonhost nests, but hungry caterpillars may also secrete their own compounds to attract ant attention, only to find that these do not mimic those of nonhost species' societies, hence identifying them as intruders at a stressful time when the colony is xenophobic.

We suggest that the additional chemical synthesis by *M. rebeli* after integration into nests of the host *M. schencki* is related to its position in the colony's hierarchy, and aimed at out-competing ant larvae for the attention of the workers. Thomas et al. (1998) showed that *M. rebeli* caterpillars are treated equivalent to *Myrmica* pupae after only 72 hr in the colony. Future studies will test whether those compounds synthesized by *M. rebeli* caterpillars after entering the ant colony that were not found on ant larvae or workers, are instead "markers" for high caste *M. schencki* individuals, namely pupae, queens, or males.

Finally, there is increasing evidence that *M. alcon* and *M. rebeli* are locally specific to different *Myrmica* ant species in different parts of their European range (Als et al., 2002; Steiner et al., 2003). Although some field samples are small, and our results here suggest that caterpillars may survive in some "nonhost" colonies in years or on sites that are exceptionally favorable, current evidence suggests that the main host of *M. rebeli* in Poland is *M. sabuleti* rather than *M. schencki* (Steiner

et al., 2003). While it will be interesting to find out whether the differences in host use are reflected in the preadoption hydrocarbon signatures, we predict that they should be more clearly reflected on the postadoption caterpillars.

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EFFECT OF FLAVONOIDS ON FEEDING PREFERENCE AND DEVELOPMENT OF THE CRUCIFER PEST *Mamestra configurata* WALKER

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Abstract—Thirty-seven flavonoid compounds (9 flavones, 18 flavonols, 8 flavanones, and 2 flavanonols) were investigated for their effect on feeding choice with bertha armyworm (*Mamestra configurata* Walker; BAW). Feeding choice was dependent upon subtle differences in biochemical structure. Unsubstituted flavone and flavanone were the strongest feeding deterrents in the choice bioassay, while 7,4′-dihydroxyflavone and dihydroquercetin stimulated BAW to feed. The constitutive flavonoids of *Brassica napus*, isorhamnetin-3-sophoroside-7-glucoside and kaempferol-3,7-diglucoside, were effective deterrents when supplemented at concentrations higher than endogenous levels. In a no-choice bioassay, flavone reduced both larval weight as well as larval and pupal development time.

Key Words—*Mamestra configurata* Walker, bertha armyworm, flavonoids, feeding deterrents, feeding stimulants, *B. napus*.

INTRODUCTION

The coevolution of plants and insects has resulted in different plant defense mechanisms, one of which is the accumulation of flavonoids that may affect feeding behavior or growth of insects (Ehrlich and Raven, 1964). Flavonoids are present in all higher plants, constituting only one group of the many metabolites that affect feeding responses of insects (Harborne, 1979). For example, resistance of soybean,

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Glycine max L., to the cabbage looper, *Trichoplusia ni* (Hübner), appears to be due to the presence of leaf flavonoids including daidzein, glyceolin, sojagol, and coumestrol (Sharma and Norris, 1991). Elliger et al. (1980), Isman and Duffy (1982), and Abou-Zaid et al. (1993) demonstrated the potency of flavonoids against growth of corn earworm *Heliothis zea* [*Helicoverpa zea* (Boddie)] larvae and the European corn borer *Ostrinia nubilalis* (Hubner) larvae. Butterflies sequester flavonoids into their wings, where they act as visual attractants to males (Gueder et al., 1997). Several studies have highlighted the dual nature of specific flavonoids as both pest-feeding deterrents and stimulants. For example, Matsuda (1978) showed that quercetin, quercitrin, rutin, myricetin, myricetrin and morin (3,5,7,2',3'-pentahydroxyflavone) all stimulated feeding by the imported willow leaf beetle *Plagiodera versicolora* (Laicharing), but inhibited feeding by the leaf beetles *Phaedon brassicae* (Baly) and *Oulema oryzae* (Kuwayama). Flavonoids have also been implicated as plant compounds that stimulate oviposition (Simmonds, 2001).

Bertha armyworm, *Mamestra configurata* (Walker) (Lepidoptera: Noctuidae; BAW), is a polyphagous insect and a serious pest of canola (*Brassica napus* L. and *B. rapa* L.), Canada's largest oilseed crop. BAW feeds vigorously on leaves, pods, and seeds of these plants, but is moderately inhibited from feeding on other types

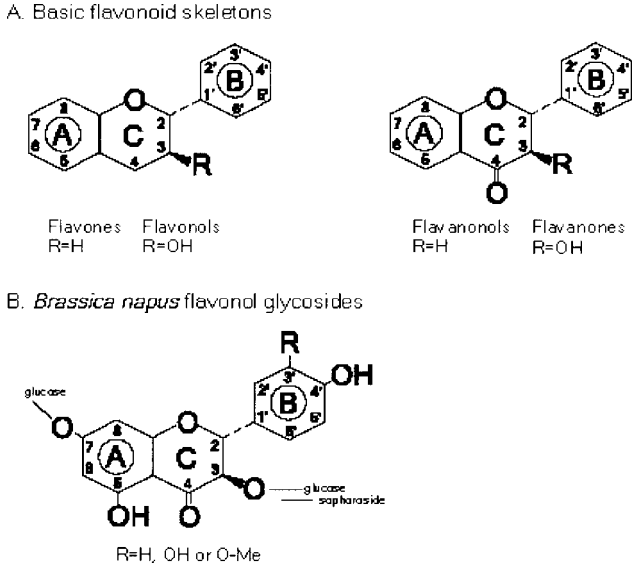

FIG. 1. Structures of parent skeleton flavonoids and *B. napus* flavonoids used. *C*-rings of flavones and flavonols are unsaturated, while those of flavanones and flavanonols are saturated.

TABLE 1. FLAVONOID COMPOUNDS TESTED IN DUAL CHOICE ASSAYS WITH BERTHA ARMYWORM LARVAE

Compounds	FDI[a]	LSD grouping[b]	*N*
Hydroxy-flavones			
1. Flavone	NA[c]		
2. 5-Hydroxyflavone	21.72	abcdefg	15
3. 7-Hydroxyflavone	1.71	cdefgh	15
4. 4′-Hydroxyflavone	28.94	abcdef	35
5. Chrysin (5,7-dihydroxyflavone)	11.81	cdefgh	15
6. 7,4′-dihydroxyflavone	−22.93	ij	15
7. Apigenin (5,7,4′-trihydroxyflavone)	1.27	fghij	15
8. 3′,4′,7-trihydroxyflavone	16.35	abcdefg	20
9. Luteolin (5,7,3′,4′-tetrahydroxyflavone)	5.06	defghi	15
Flavanones and flavanonols			
10. Flavanone (2,3-dihydroflavone)	40.69	ab	15
11. Naringenin (5,7,4′-trihydroxyflavanone)	−14.53	hij	15
12. Naringin (naringenin-7-0-neohesperidose)	35.89	abc	15
13. 5-Methoxyflavanone	0.86	fghij	15
14. 6-Hydroxyflavanone	−0.58	fghij	15
15. Eriodictyol (5,7,3′,4′-tetrahydroxyflavanone)	19.02	abcdefg	15
16. Hesperetin (5,7,3′-trihydroxy-4′-methoxyflavanone)	−13.36	gij	15
17. hesperidine (hesperetin-7-0-rutinoside)	8.11	cdefgh	15
18. Dihydrokaempferol (3,5,7,4′-tetrahydroxyflavanone)	2.71	fghij	26
19. Dihydroquercetin (3,5,7,3′,4′-pentahydroxyflavanone)	−28.75	j	15
Flavonols			
20. Flavonol (3-hydroxyflavone)	−7.58	ghij	30
21. 5-Deoxykaempferol (3,7,4′-trihydroxyflavone)	17.7	abcderg	15
22. Kaempferol (3,5,7,4′-tetrahydroxyflavone)	3.09	fghij	30
23. Kaempferol-3-0-glucoside	17.78	abcdefg	45
24. Kaempferol-3-0 –rhamnoside	26.78	abcdef	30
25. Kaempferol-3-0-rutinoside	13.35	bcdefgh	30
26. Kaempferol-4′-0-methyl ether	22.13	abcdefg	20
27. Fisetin (5-deoxyquercetin)	5.59	fghij	15
28. Quercetin (3,5,7,3′,4′-pentahydroxyflavone)	−8.48	ghij	30
29. Quercetin-3-0-glucoside	11.77	cdefgh	30
30. Quercitrin (quercetin-3-0-rhamnoside)	35.08	abc	20
31. Rutin (quercetin-3-0-rhamnoglucoside)	−5.05	ghij	30
32. Isorhamnetin (quercetin-3′-0-methyl ether)	24.10	abcdefg	15
33. Myricetin (3,5,7,3′,4′,5′-hexahydroxyflavone)	32.44	abcde	15
34. Myricitrin (myricetin-3-0-rhamnoside)	−7.18	ghij	20
B. napus extracted flavonols[d]			
35. Kaempferol-3,7-0-diglucoside	20.81	abcdefg	15
36. Kaempferol-3-0-sophoroside-7-0-glucoside	−2.67	ghij	14
36. Isorhamnetin-3-0 -sophoroside-7-0-glucoside	44.76	a	14

[a] FDIs $\leq +20\%$ and $\geq -20\%$ encompass the "no useful response" range, such that the corresponding compounds would not contribute substantially to *B. napus* resistance/susceptibility to BAW in a canola crop setting.

[b] Feeding-deterrence indices under the same lower-case letter were not significantly different (one-way ANOVA of ranked data, $P > 0.05$).

[c] NA, not applicable.

[d] Extracted from *B. napus* 3-wk-old leaves.

of crucifers, such as several types of *B. juncea* L. and *Sinapis alba* L. cultivars and breeding lines (Ulmer, 2002). During an outbreak year, crop damage soars from late July to mid-August when the insects molt to the fourth (second-last) instar. Larvae in the last two larval stages consume 80–90% of the plant material necessary to complete their life cycle.

In this study, we investigated the ability of flavonoids to stimulate or deter the feeding behavior of BAW, because of their ubiquitous distribution in the Brassicaceae (Onyilagha, 2003). A wide range of commercial flavonoids were tested to determine whether there are specific flavonoid structures that influence BAW feeding and that potentially could be introduced into *B. napus* from wild or domesticated crucifer relatives. We also conducted experiments to determine the contribution of endogenous flavonoids in *B. napus* to BAW feeding patterns and to assess the potential for *B. napus* to protect itself against BAW if the endogenous flavonoid pathway is stimulated.

METHODS AND MATERIALS

Insect Rearing. Early 3rd-instar BAW larvae were obtained from a colony maintained at the Saskatoon Research Centre. Larvae were raised from eggs and reared on an agar-based artificial diet (Bucher and Bracken, 1976).

Flavonoid Preparation. Commercial compounds were obtained from Apin Chemicals (UK) or Sigma (Canada) and dissolved in either 95% ethanol, methanol, aqueous solutions of the previous solvents, or chloroform.

Leaf flavonoids from the host plant, *B. napus* var. AC Excel, were extracted and quantified using standard procedures as previously described (Harborne, 1998). Briefly, flavonoid aglycones were extracted by boiling 100–200 g cut tissue in 2N HCl for 30 min. Extracts were allowed to cool and aglycones were extracted into ethyl acetate. Ascending chromatography (1-D) was carried out on cellulose, nonfluorescent plastic TLC plates in three different freshly prepared solvent systems: butanol–HOAc–water (4:1:5, v:v:v, top layer); Forestal [HCl–HOAc–H_2O (3:30:10, v:v:v)]; and HOAc–H_2O (1:1, v:v). *B. napus* flavonoid aglycones were separated alongside authentic quercetin, kaempferol, and isorhamnetin standards and identified by comparing with published values their relative mobility (R_f) values and color under UV light (before and after fuming with NH_3 vapor) (Harborne, 1967, 1998; Mabry, 1970). Flavonoid aglycones were also identified by chromatography on a 5 μ Zorbax SB-C18 semipreparative column (9.4 $\times$ 250 mm) using an Hewlett Parkard 1100 series HPLC equipped with a diode array detector. Peaks were monitored at 220, 254, and 300 nm. The following 60-min gradient was used at a flow rate of 3 ml/min: t_0, 100:0; t_{10}, 85:15; t_{50}, 72:28; and t_{60}, 0:100 (water/acetonitrile, respectively).

Kaempferol-3,7-diglucoside, kaempferol-3-sophoroside-7-glucoside, and isorhamnetin-3-sophoroside-7-glucoside were extracted from 3-wk-old *B. napus* leaves according to Harborne (1998). Leaves (200 g) were extracted overnight with 100% MeOH and the extracts subjected to 2-D chromatography, first in butanol–HOAc–water (4:1:5, top layer) and then in 15% HOAc in water. Flavonoid glycosides were identified using the same criteria as for their aglycones. Individual flavonoids were separately scraped off the TLC plate, eluted with 80% MeOH into a preweighed vial, dried *in vacuo*, and reconstituted in 80% MeOH.

Exudate flavonoids (deposited onto the leaf surface) were extracted from 3-wk-old leaves (70 g) using a slight modification of the method of Harborne et al. (1994). Whole leaf tissues were carefully rinsed (3x, about 10–15 sec each) in dichloromethane (DCM), and concentrated to dryness *in vacuo*. The white DCM exudate was reconstituted and chromatographed on silica gel G plates using a mixture of toluene–acetic acid (4:1). Chromatograms were viewed under UV light in the presence of NH_3 vapor.

Choice-Feeding Bioassay. The feeding response of BAW to commercially available flavone was assayed in a leaf disk binary choice test (Isman et al., 1990; Dimock et al., 1991). Bioassay dishes (22-mm deep, 40-mm diam.) were lined with Whatman No.1 filter paper, wetted with deionized water (180 μl) to maintain humid conditions and to prevent leaf disk dehydration. Pairs of disks (10-mm diam, 20 mg ($\pm$ 0.1) (SE) were cut with a No. 5 cork borer from the same region of 4-wk-old leaves of greenhouse-grown *B. napus* var. AC Excel. Leaf disks cut from 4-wk-old plants lay flat (facilitating the application of extracts), had the smallest increase over 24 hr (observed by a Pseudocolor AgVision digital imaging analysis system [DIAS]), and the leaves at this age were well expanded. A range of flavone doses (0.05–0.45 μmol per leaf disk) was applied evenly in an ethanol-carrier solution onto the upper surface of each treatment leaf disk in 10-μl aliquots. This dose range was the equivalent of an endogenous fresh weight (FW) leaf disk concentration ranging from 0.05–0.5% (% dose per FW leaf disk, a calculation used to assess potential for developing a chemical as an endogenous feeding deterrent in *B. napus* through breeding, i.e., potentially useful if moderately to strongly deterrent at $<$0.1% FW). Ethanol-carrier control disks in each pair received 10-μl ethanol to control for and monitor differences in leaf structure that might influence insect feeding, leaf hydration, or leaf disk size. The ethanol was allowed to evaporate completely before putting disks into the assay dishes. One flavone treatment disk and an ethanol-carrier control disk were placed directly opposite each other in each treatment choice bioassay dish. Control choice bioassay dishes included one ethanol-carrier leaf disk and a leaf disk free from chemical application as a means to determine the extent to which the carrier affected feeding.

Five 3rd-instars of BAW were released at the center of each prepared dish (arena). Dishes were covered with lids (but not sealed), leaving the larvae to choose between treatment and control disks. Dishes were incubated for 20 hr at 21°C +/-1,

98% RH +/-1 (Koul and Isman, 1992). At the end of the bioassay period, each disk was digitized and the area consumed was determined. The area of control disks typically fed upon during this time was <70%, but ranged between 45 and 85% of the disk area depending on the cohort insects. Feeding was not allowed to progress to the point that BAW did not have a choice of disks. A feeding deterrence index (FDI), $[(C - T)/(C + T)] \times 100\%$, was calculated for a range of flavone concentrations, where C is the area consumed for the control disks and T is the area consumed for the treatment disks (Koul and Isman, 1992).

Based on this feeding study, the dose of flavone required to give a 50% feeding deterrence index (DC_{50}) with 3rd instars was calculated. This dose (0.1 μmol per disk, equivalent to a hypothetical endogenous concentration of 0.12% FW^{-1} leaf disk) and a similar experimental design were subsequently used to test the feeding activity of BAW on a broad range of commercial flavonoids in paired-disk bioassays (Table 1; Figure 1). Solvents that did not strongly affect leaf disk structure and area (i.e., curling, swelling) were chosen to dissolve these flavonoids. Paired-control assays were set up to test the effect of these solvents on BAW feeding compared with water. A negative FDI as calculated above was interpreted as feeding stimulation, while a positive index was regarded as deterrence. Flavonoid aglycones identical to those determined for the host plant, *B. napus*, and that could be purchased commercially, were tested at the DC_{50} dose determined for flavone, as well as at a four-fold higher dose. Flavonoid glycosides extracted from *B. napus* and unavailable commercially were only tested at the higher dose. Each assay was replicated two or three times using different cohorts of insects and 5–15 arenas within each replication. Feeding indices were calculated separately for each arena. Indices $\leq +20\%$ and -20% FDI were considered within a "no useful response" range, were not plotted, since the corresponding compounds would not contribute substantially to *B. napus* resistance to BAW in a canola crop setting.

No-Choice-Feeding Bioassay. Flavone was dissolved in 95% ethanol/H_2O (1:1, v/v), incorporated into cooled liquid artificial diet (Bucher and Bracken, 1976) at concentrations ranging from 100–200 $\mu g\, g^{-1}$ diet, and ~6.3 g diet was allowed to solidify in diet cups similar in size to the choice arenas. Control diets with equivalent amounts of ethanol-carrier or unsupplemented diet were also prepared to control for ethanol-induced changes in insect physiology. Empty diet cups and solidified diet surfaces were lightly misted with fungicide (2.4 g methyl benzoate – 6 g sorbic acid in 400 ml 95% EtOH). Two, early 3rd-instar BAW (~6.2 mg each) were introduced into each diet cup, and covered with 1.55-mm unwaxed paper disk caps (15 cups for each treatment or control diet). Combined larval weights and developmental stages for each cup were recorded every 48 hr until pupation. Insects were transferred to fresh diet cups every 48 hr to prevent desiccation and food deprivation. Upon pupation, the number of larvae that pupated, pupal weights for each sex, pupation time, adult moth weights for each sex, and adult activities

were recorded. Pupation (survival) rate among larvae on control diets ranged from 60 to 100% of initial larvae in the trial and was dependent upon the overall health dynamics within the colony at the time of testing.

Insect development as a function of dietary flavone concentration was analyzed by analysis of variance and linear regression (SAS, 1999). A *t*-test (least-squares difference) was applied to ranked FDIs of commercial and extracted flavonoids (SAS, 1999). [The *F* test generated by the parametric procedure when applied to ranks is as valid an assessment as the χ^2 approximation utilized by Kruskal–Wallis (SAS, 1999)].

RESULTS AND DISCUSSION

Feeding Deterrence or Stimulation of Commercial Flavonoids. Flavone significantly decreases survival and mean weight of nymphs of the migratory grasshopper, *Melanopus sanguinipes* (Fabricius) (Westcott et al., 1992) and deters feeding by the beet armyworm, *Spodoptera frugiperda* (J. E. Smith) (Wheeler et al., 1993). These multiple effects on vigorous generalist insect feeders led us initially to choose flavone to begin our investigation of the effects of flavonoids on BAW. BAW larval response to increasing doses of flavone (Figure 1; Table 1, compound 1) in a choice binary feeding assay is illustrated in Figure 2. Typically, BAW larvae did not discriminate between untreated or ethanol-carrier control leaf disks. At

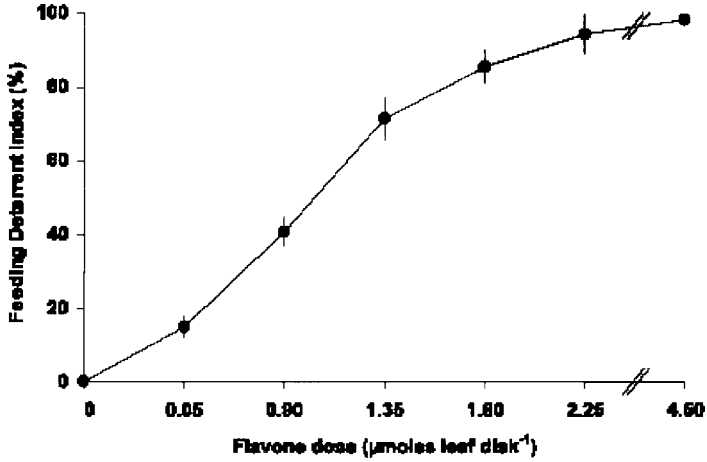

FIG. 2. Dose-response of BAW larvae exposed to flavone in a leaf disk choice-feeding bioassay. Control disks were treated with ethanol used to dissolve the flavone. Vertical lines indicate standard error of the means. Double lines indicate interruption of scale.

doses up to 0.14 μmol per disk, the amount of feeding was inversely proportional to the concentration of flavone. Above 0.18 μmol per disk, BAW avoided all treatment disks. The dose of flavone determined to give a 50% feeding deterrence index (DC_{50}) with BAW was 0.1 μmol per disk. This dose is equivalent to a hypothetical endogenous concentration of 0.12% FW and one that could provide a strong level of insect deterrence without likely compromising plant productivity and growth.

In a corresponding no-choice-feeding bioassay, approximately 29 and 53% of the leaf disk area was not consumed by BAW larvae when treated with 0.1 and 0.14 μmol doses of flavone, respectively. This no-choice experiment suggested that BAW larvae could feed on foods containing flavone, although they would normally try to avoid diets containing this compound. Subsequently, 3-wk-old larvae were fed a diet supplemented with flavone in a no-choice experiment to determine whether flavone could have a toxic effect on BAW growth and development in addition to deterrent properties if the food choice was limiting. Diets supplemented with 100 μg flavone g^{-1} diet (equivalent to 0.01% FW diet) had no effect on BAW larval weight, rate of weight gain, or insect development patterns compared with plain diet or diet supplemented with ethanol-carrier (Figure 3 A and B). Larval survival remained at 100% for the flavone treatment, 97% for ethanol-supplemented diet, and 100% for unsupplemented diet. In contrast, the rate of weight gain on a 200 μg flavone g^{-1} diet was reduced from 5.7 to 1.4 mg day $^{-2}$. This reduction was so extreme that development time for 4th instars to enter the 5th and 6th instar stages and pupal formation was delayed by 6 days (Figure 3 C and D), and the time to complete larval development was extended to 18–30 days compared with the usual 14–22 days on control diets. Thirty percent of larvae died within the first 8 days of the 200-μg flavone trial, and total larval survival was reduced to 43% compared with the ethanol-supplemented (93%) and control diets (100%). Poisoned larvae typically congregated off the diet surface and appeared black and flaccid shortly before death, similar to patterns of normal larval death. Maximum 6th instar weight achieved by surviving larvae just prior to pupation was equivalent for all the diets regardless of the time it took to achieve the maximum (685.8 $\pm$ 32.1 mg). Flavone is highly toxic to young BAW larvae at a five-fold lower dose than the DC_{50} if their food choice is restricted.

Since exposure to 200-μg flavone severely affected mortality and growth rate of BAW larvae, sublethal damage was examined in pupae and adults emerging from a diet supplemented with 150 μg flavone g^{-1} diet. Under these growth conditions, the rate of larval weight gain was reduced to 75% of control diets, development of the 5th and 6th instars was delayed by 2 days, initiation of pupation was delayed by 4 days, but survival was not affected (data not shown). Pupation time appeared normal under these sublethal conditions [26.2 days ($\pm$0.8), regardless of the sex and diet]. Pupal weight was affected by gender ($P \leq 0.001$), but not by diet, with female pupae consistently heavier than male pupae [378 mg ($\pm$16.8) and 326.0 mg ($\pm$10.6), respectively]. Sublethal damage did not appear to affect adult males or

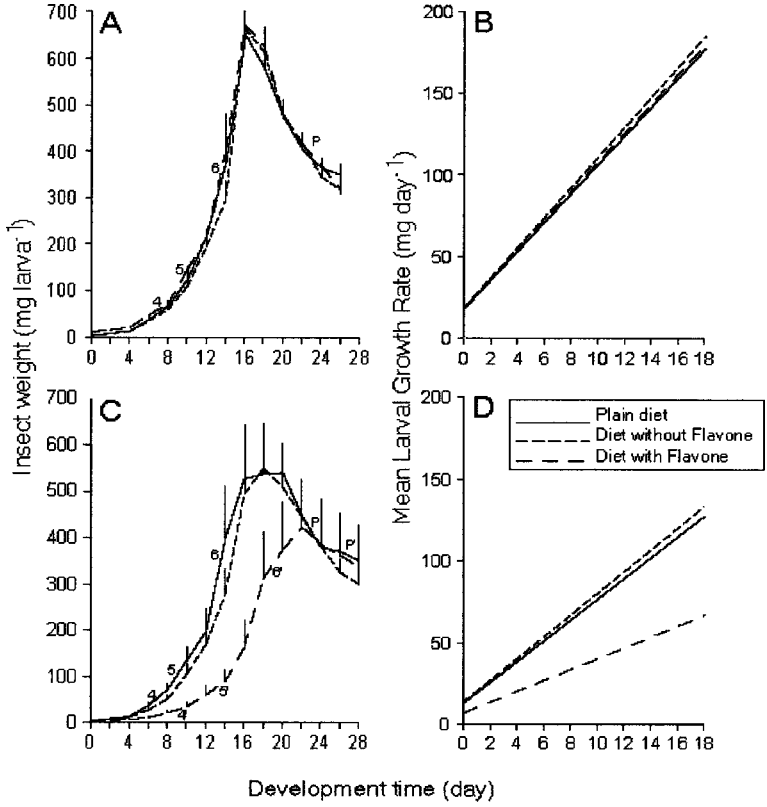

FIG. 3. Effect of flavone on the growth and development of third-instar BAW in a no-choice-feeding assay. A and B: 100 μg flavone g^{-1} diet; C and D: 200 μg flavone $^{-1}$g diet. A and C: Profile of average insect weight during larval and pupal developmental stages. The occurrence of larval stages (1–6) and the pupal stage (P) is indicated for plain (unsupplemented) diet and ethanol-carrier-supplemented diet without flavone (same unprimed number for both types) and flavone-supplemented diet (primed number). B and D: Mean larval growth rate. Flavone was incorporated into an artificial diet in an ethanolic-carrier solution. Vertical lines in panels A and C indicate standard error of the means (positive mode only). Several small error bars are masked by larger ones.

females, although female moths weighed more than male moths [190.2 mg ($\pm$18.2) and 150.1 mg ($\pm$10.8), respectively]. All emerging moths assumed normal activity regardless of diet, i.e., moving around easily, displaying strong flying activity, and eagerly searching for food. These data suggest that sublethal damage from flavone was limited to the larval stage of development. We did not examine fecundity in the adults.

No-choice-feeding experiments suggest that BAW cannot complete a life cycle efficiently in the presence of flavone. However, mode-of-action of deterrence and toxicity in BAW larvae is not established. Flavone induces polysubstrate monooxygenases, general esterases, and glutathione *S*-transferases in the gut of fall armyworm [*Spodoptera fugiperda* (J. E. Smith)] (Yu, 1986; Wheeler et al., 1993). These enzymes enable fall armyworm to metabolize flavone, although this insect still avoids plants known to accumulate this compound. We have not determined whether these same enzymes are induced by flavone in BAW gut. However, the inability of BAW larvae to grow normally when forced to eat small amounts of flavone suggests that BAW may not be equipped with detoxifying enzymes necessary for constant exposure to this compound in its food. Indeed, sequences for several of the detoxifying enzymes induced in fall armyworm by flavone do not appear in a noninduced cDNA library of BAW gut (Hegedus, unpublished).

The initial choice and no-choice studies with flavone support the hypothesis that flavonoids can affect feeding of BAW. However, flavonoids usually accumulate as derivatives in plants (Harborne, 1988), including in several crucifer species with potential to transfer unique flavonoid genes into canola in a breeding program. For example, flavone glycosides accumulate in *Thlaspi arvense* and *Crambe abyssinica* (Onyilagha et al., 2003), and flavonol glycosides accumulate in *S. arvensis* (Durkee and Harborne, 1973), although the basis of their resistance to BAW has not necessarily been confirmed. Indeed, a broad survey indicates that flavonols accumulate in most of the Brassicaceae, although flavones have been found in a few additional genera: *Alliaria, Alyssum, Barbarea, Capsella, Lepidium, and Raphanus* (Onyilagha et al., 2003); and flavanones have been found in *Mattiola* (Chapman and Hall, 1982–98).

In order to determine the types of flavonoids, in addition to flavone, that have potential to influence BAW feeding on Brassicaceae, a range of commercial flavonoids (detailed in Table 1) were tested in BAW choice-feeding bioassays. These compounds were bioassayed at the same dose that caused 50% feeding deterrence by flavone, i.e., 0.1 μmol per disk. Many of the compounds were tested because they contained flavonoid structural features that are found in Brassicaceae species with potential for interspecific or intergeneric gene transfer (Fahleson et al., 1994; Youping and Peng, 1998), although the specific derivatives found in these crucifers were not necessarily available commercially nor have all of their structural features been determined (Durkee and Harborne, 1973; Onyilagha et al., 2003). Others were tested because of subtle differences in structure that had the potential to influence BAW feeding, and required evaluation for potential utility in a crop-breeding program.

In general, when the basic flavone skeleton is substituted with either hydroxyl groups or *O*-glycosides on any part of the molecule, bioactivity is reduced (Table 1). Hydroxylation at C_3 to create unsubstituted flavonol (Table 1, compound 20) appears to reduce feeding deterrency more than any other single substitution

event, changing the FDI to −8%. However, the relationship between bioactivity and oxidation pattern is less clear in BAW than in reports where highly oxidized coumestans or isoflavones deterred *Costelytra zealandica* (White) larvae (Lane et al., 1985), when two oxidized flavonoids, quercetin (compound 28) and rutin (compound 31), deterred adult striped cabbage flea beetles [*Phyllotreta striolata* (Fabricius)] or specialist pests of cruciferous species were also deterred (Figure 1) (Meisner and Mitchell, 1984). High *C*-ring oxidation as a factor that promotes stimulation of BAW feeding contrasts with the increase in antifeeding activity when flavonoids with *B*-ring oxidation were fed to the European elm bark beetle *Scolytus multistriatus* (Marsham) (Norris, 1977), a species that is unrelated to BAW.

BAW feeding response to substituted commercial flavonoids did not depend upon their hydrophilic or hydrophobic nature. Tests with a range of structures in which the $C_2{=}C_3$ bond was reduced to $C_2{-}C_3$ (as in flavanones) or reduced and hydroxylated at C_3 (as in flavanonols) indicated several associations between substitution pattern and bioactivity that could be detected by LSD analysis of ranked FDIs (Table 1; Figure 1) (SAS, 1999). The unsubstituted flavanone (compound 10) (Table 1) was almost as effective at deterring BAW feeding as flavone (compound 1) (Figure 2), while C_3 hydroxylation promoted little or no feeding when presented in most substituted flavanonols, for example in dihydrokaempferol (compound 18). However, flavones, that are mono-hydroxylated at either C_5 or $C_{4'}$ positions (compound 2 and 4, respectively) mildly deterred BAW (to a degree that would be useful in crop breeding) compared with other substituted flavones, whereas 7,4′-dihydroxyflavone (compound 6) and dihydroquercetin (compound 19) mildly stimulated BAW to feed.

Since flavonols are the most common flavonoids found within the Brassicaceae (Durkee and Harborne, 1973; Nielson et al., 1993, 1998; Greenberg et al., 1996; Olsson et al., 1998; Wilson et al., 1998; Onyilagha et al., 2003), a much larger number of flavonol derivatives were tested for their effects on BAW feeding than other flavonoid structures. Most substituted flavonols weakly changed BAW normal feeding pattern on *B. napus* or were completely ineffective (Table 1, Figure 1). However, several flavonols were mild feeding deterrents, for example myricetin (compound 33), which has a full, but unsubstituted hydroxylation pattern. Curiously, attaching a single rhamnose moiety at the C_3 hydroxyl of myricetin to form myricitrin (compound 34) resulted in a compound that was completely ineffective as a BAW-feeding deterrent. This type of substitution had the opposite effect when applied to quercetin (compound 28) and kaempferol (compound 22), which differ from myricetin only by their hydroxylation pattern on the B-ring (Table 1, Figure 1), turning quercetin into the moderately effective deterrent quercitrin (compound #32). A deterrent relationship was also observed when quercetin-3-rhamnoside, rather than quercetin-3-glucoside, was incorporated into artificial diet and fed to silkworm, *Bombyx mori* (Linnaeus) larvae (Harborne, 1979). Our data demonstrate the importance of flavonoid specificity (i.e., group

and position substitution) to BAW, as suggested for insects as a whole by Harborne (1979). In doing so, they draw attention to the value of determining the exact flavonoid substitution pattern in crucifer germplasm used in an insect-resistance-flavonoid selection program, rather than relying on the more easily analyzed aglycone.

Methoxylation of the *B*-ring of quercetin and kaempferol produced a mild deterrent response by BAW, for example when BAW was exposed to isorhamnetin (compound 32) or kaempferol-4'-O-methyl ether (compound 26) (Table 1). These data, together with the increase in deterrence when the C_3 hydroxyl of quercetin was monosubstituted, suggest that the substitution pattern associated with or near the flavonoid B-ring may be important to BAW's response to food. Potentially, glycosylation or sulfation of specific hydroxyl groups at these locations could play a role in BAW's ability to metabolize quercetin, as part of a metabolite detoxification scheme present in insects (Yu, 1986). In contrast, *B*-ring *ortho*-hydroxylation improves the efficacy of flavonoids as an antifeedant for the closely related noctuid corn earworm *Heliothis zea* [*Helicoverpa zea* (Boddie)] larvae (Elliger et al., 1980).

Feeding Deterrence of Brassica napus *Flavonoids.* Leaf tissues are the main food source for BAW in canola fields and the most common substrate for egg-laying. *Brassica napus* accumulates low quantities of several leaf flavonol glycosides similar to those in *S. arvense* and *S. alba* (Durkee and Harborne, 1973; Nielsen et al., 1993, 1998; Greenberg et al., 1996; Olsson et al., 1998; Onyilagha et al., 2003), two species which are resistant to BAW (Ulmer, 2002). However, the role of endogenous *B. napus* flavonoids in BAW feeding stimulation and their potential for development as deterrents is unknown. At the time of this experiment, most *B. napus* compounds were not available from commercial sources, and it was unclear whether the quantity or composition of flavonoid classes in *B. napus* changed with plant development. Hence, TLC analysis of acid-hydrolyzed leaf extracts of *B. napus* var. AC Excel was conducted to profile the variability of specific flavonoid classes from the first true leaf stage to the sixth leaf stage. Feeding bioassays were conducted on *B. napus* extracted flavonoids.

Flavonoid profiling indicated that kaempferol was the most abundant type of flavonoid present in leaves at all stages of plant development (Table 2). Isorhamnetin was barely detectable in leaves at the second stage and peaked at trace levels by the fourth stage. Quercetin was easily detected in the second stage, but declined to trace levels in more mature leaf tissue. Subsequently, quantification of flavonoid classes was conducted in acid-hydrolyzed extracts of 3-wk-old leaves (between the second and third leaf stage of plant development), a stage that normally would not sustain damage by 3rd-instar BAW in a field infestation, but which accumulated flavonols of all classes. At this stage, kaempferol was accumulated at 150 $\mu g\, g^{-1}$ FW tissue (0.015% FW), quercetin at 5.86 $\mu g\, g^{-1}$ FW tissue (0.0006% FW), and isorhamnetin at 1.66 $\mu g\, g^{-1}$ FW tissue (0.0002% FW). Lipophilic flavonoids, i.e., flavonol aglycones and highly methylated glycosides (Wollenweber and Jay, 1988), were not found in dichloromethane washes of *B. napus* leaves. The low

TABLE 2. FLAVONOID COMPOSITION IN DEVELOPING *B. napus* LEAVES

True leaf stage	Days after planting	Flavonoids in hydrolyzed leaf extracts[a]		
		Kaempferol	Quercetin	Isorhamnetin
1	13	+++	−	−
2	19	+++	++	+
3	26	+++	+	−
4	31	+++	+	++
5	38	+++	+	+
6	46	+++	+	++

[a] +++, Bright yellow on TLC plate indicating relative abundance, ++, faded yellow on TLC plate indicating lower levels than kaempferol, +, trace levels only.

content of flavonols during development in var. AC Excel suggests that they do not influence BAW feeding at their endogenous level. However, the total phenolic content of *B. rapa* foliage with advancing age has been correlated with an increase in BAW feeding (McClosky and Isman, 1995). A rigorous examination of the content, composition, and insect feeding preferences of all phenolics in *B. napus* as a function of tissue type and developmental stage, in addition to flavonoids, should provide greater understanding of the role the phenolic family of plant secondary metabolites plays in insect pest–host plant interactions in this species.

In order to predict whether *B. napus* endogenous flavonoids are stimulants to BAW or have potential as deterrents, paired leaf disks were spread with purified flavonols extracted from 3-wk-old true leaves of var. AC Excel. Since structural specificity rather than flavonoid backbone *per se* is important to BAW, three leaf flavonol glycosides were purified from 3-wk-old bulked plantings of *B. napus* var. AC Excel by preparative 2-D TLC, eluted with 80% MeOH, concentrated, and applied at 4 μmol per leaf disk as previously described. Initially, the two major kaempferols were extracted and tested, since this type of flavonol structure accumulated in a 30-fold higher concentration in *B. napus* leaves compared with other flavonols (outlined earlier). Kaempferol-3,7-diglucoside (compound 35) tested at this concentration provided a low amount of deterrence against BAW (Table 1), while supplementation with kaempferol-3-sophoroside-7-glucoside (compound 36) did not provide added protection to the treatment disk (Table 1).

Since quercetin only accumulated in trace amounts in var. AC Excel leaves and only two commercial quercetin derivative (compound 30 and 32) out of four tested had a deterrent effect on BAW, quercetin derivatives were not extracted and tested in the disk bioassay. Instead, isorhamnetin, which accumulated in trace amounts in *B. napus*, as well as in *S. arvense* and *S, alba* (Durkee and Harborne, 1973), was extracted as isorhamnetin-3-sophoroside-7-glucoside (compound 35) from *B. napus* leaves. When applied to treatment leaf disks, this compound was as deterrent to BAW feeding as flavone at the DC_{50} dose (Table 1). This suggests that *B.*

napus does contain the innate potential to develop a moderate degree of resistance to BAW, if the concentration of kaempferol-3,7-diglucoside and isorhamnetin-3-sophoroside-7-glucoside could be increased. This also supports earlier speculation, based on studies with commercial flavonoids, that the presence of a methoxy group on the *B*-ring of the quercetin frame and substitution of the hydroxyl at C_3 may play a role in BAW feeding deterrence or the prevention of detoxification by BAW.

In summary, although commercial flavone was the strongest deterrent to BAW of all the commercial flavonoids tested, several flavonoid derivatives, including flavanone, naringin, quercetrin, and myricetin also exhibit moderate deterrence to BAW feeding, while dihydroquercetin and 7,4-dihydroxy-flavone stimulate BAW to feed. The number of flavonoid derivatives tested was not exhaustive, but the fact that BAW responds to flavonoids in a selective fashion suggests that these compounds, in addition to glucosinolates (Hicks, 1974; Reed et al., 1989; Bodnaryk, 1991; Roessingh et al., 1992; Larsen et al., 1992), have the potential to influence BAW feeding in crucifer germplasm where they are present in significant quantity. This would include *Thlaspi arvense*, the non-cultivated species that accumulates significant quantities of a flavone glycoside (Onyilagha et al., 2003). Resistance to BAW could be achieved in *B. napus* by introducing a regulatory gene that stimulates ectopic accumulation of endogenous flavonols and their specific glycosyl transferases. One such gene could be isolated from a UV-responsive differential cDNA library, since kaempferol glycosides and quercetin glycosides (but not isorhamnetin glycosides) accumulate under enhanced UV light in leaves of *B. napus* cv. Tobas (Wilson et al., 1998). Alternatively, chemically mutagenized *B. napus* lines, that have been developed at the Saskatoon Research Centre (Raney, unpublished), could be screened to select a mutant in which isorhamnetin glycoside accumulated under normal growth conditions. Given the recent efforts in crucifer genomics world-wide (Arabidopsis Genome Initiative, 2000; Huala et al., 2001), such genes or mutants could be available within the next few years. Their use in germplasm development could lead to resistance to BAW in the Canadian canola crop.

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SIGNIFICANCE OF WOOD TERPENOIDS IN THE RESISTANCE OF SCOTS PINE PROVENANCES AGAINST THE OLD HOUSE BORER, *Hylotrupes bajulus,* AND BROWN-ROT FUNGUS, *Coniophora puteana*

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Abstract—We tested how terpenoid (i.e., monoterpenes and resin acids) composition and concentration in wood affects resistance against wood-borers and decaying fungi. Scots pine (*Pinus sylvestris*) wood from nine provenances having variable terpenoid profiles was studied against the old house borer, *Hylotrupes bajulus,* and the decay fungus, *Coniophora puteana*. Provenances represented a 1200-km N–S transect from Estonia to northern Finland, but they were all cultivated for 7 years in the same nursery field, in central Finland. Mean relative growth rate (MRGR) of small *H. bajulus* larvae positively correlated with the total monoterpene concentration of wood, and feeding was associated with high proportion of levopimaric+palustric acid in wood. Provenance did not affect the MRGR of small or big larvae, but big larvae consumed more wood and produced more frass on the northern Ylitornio trees than on the southern Rakvere and Ruokolahti trees. Low β-pinene and total monoterpene concentration and low β: α-pinene ratio in wood were all associated with a high number of eggs. The most northern Muonio provenance was the most favored as an oviposition site, differing significantly from Saaremaa, Tenhola, and Suomussalmi. Wood from Saaremaa, Tenhola, Ruokolahti, and Suomussalmi provenance was most

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resistant against decay fungus, differing significantly from that of Kinnula provenance. However, decay resistance was not clearly associated with the concentrations of wood terpenoids. These results suggest that monoterpene composition of wood affects resistance against wood-boring Cerambycid beetles, but resistance against wood-decaying fungi is not as clearly associated with wood terpenoids.

Key Words—*Hylotrupes bajulus*, *Coniophora puteana*, Scots pine, provenance, wood, wood extractives, resin acids, monoterpenes, wood-borer, decay resistance.

INTRODUCTION

In nature, the geographical distribution of *Hylotrupes bajulus* (L.) (Coleoptera: Cerambycidae) (old house borer, house longhorn beetle) is limited to the conifer belt of the Baltic sea area (Ljungkvist, 1983), but it has become a worldwide insect pest of construction timber. Larval stages of this insect cause considerable damage to the seasoned coniferous timber commonly used in buildings (Robinson and Cannon, 1979). Under the fluctuating environmental conditions in nature, larval development can last 10 or more years, but under optimal conditions larval development time can occur in approx. two years (Robinson and Cannon, 1979). Larval development is most affected by temperature, relative humidity, wood moisture content, and wood nutrients (especially proteins and other nitrogen containing compounds) (Berry, 1972). Larvae prefer the sapwood of pine, spruce, and fir, leaving the inner heartwood and the outermost surface of the timber intact. Larvae are incapable of using hardwood as a food source.

Conifers produce oleoresin, which consists of terpenes (mono- and sesquiterpenes) and resin acids (diterpenes), and phenolic compounds that are all known to act as feeding deterrents to a variety of generalist and specialist insects (Gershenzon and Croteau, 1991; Langenheim, 1994). Phenolic compounds (e.g., stilbenes) occur only in the heartwood, whereas resin acids are found at low concentrations also in the sapwood. Female *H. bajulus* do not accept hardwood as ovipositional sites, because hardwood does not contain suitable mixtures of terpenes (Holm and Ekbom, 1958). In wind tunnel tests, α-pinene (monoterpene) increased the activity and orientation of both male and female *H. bajulus* (Fettköther et al., 2000). On the other hand, pheromone components (−)-verbenone and *p*-cymen-8-ol produced in the frass of the wood-boring larvae of *H. bajulus* have mediated the oviposition behavior of adults (Higgs and Evans, 1978).

Phenolic compounds (e.g., stilbenes) and oleoresin compounds, especially resin acids, have important value in decay resistance of conifers (Hart and Shrimpton, 1979; Rao, 1982; Yamada, 1992; Venäläinen, 2002). Several monoterpenes (e.g., Flodin and Fries, 1978; Schuck, 1982), resin acids (Henriks et al., 1979), and phenolic compounds (Rennerfelt and Nacht, 1955) incorporated into agar inhibit the growth of wood-rotting fungi. However, it is still unclear to what

extent oleoresin compounds inhibit fungal growth on a natural substrate, such as wood. In living plants, there are trade-offs in plant defense pathways against pathogens and herbivores (Thaler et al., 1999). Decay resistance of wood has been tested, e.g., with *Coniophora puteana* (Schum.:Fr.) P. Karsten, which is a common brown rot fungus degrading only cellulose and hemicellulose, but not lignin. It is a fairly aggressive fungus (Rennerfelt, 1956) that causes important economic damage to timber, but is unable to spread into dry timber. Growth of *C. puteana* is stimulated by Scots pine volatiles (Mowe et al., 1983). The total concentration of pinosylvin and resin acids in Scots pine heartwood are higher in decay-resistant versus decay-susceptible trees (Harju et al., 2002; Venäläinen et al., 2003), and wood blocks impregnated with resin acids decay less than unimpregnated ones (Hart et al., 1975; Eberhardt et al., 1994). Resin acids belonging to the abietane group have higher fungitoxicity than resin acids of the pimarane group (Micales et al., 1994). Generally, there is high variation in the content of wood extractives, e.g., resin acids and pinosylvin, among individual trees, which to a large extent is under genetic control (Fries et al., 2000). Earlier we studied variation in needle and wood terpenoid content among nine different Scots pine provenances from a 1200-km north–south gradient (Manninen et al., 2002).

In this study, we wanted to find out if terpenoid composition and concentration in Scots pine wood can explain both resistance against a wood-boring insect and a decay fungus. The nine provenances had variable wood terpenoid composition. Specific aims were to (1) compare the mean relative growth rate of old house borer [*Hylotrupes bajulus* (L.)] larvae feeding on wood from different provenances; (2) determine the oviposition preference of *H. bajulus* adults on six selected provenances; (3) study the resistance of wood from all provenances against brown rot fungus, *Coniophora puteana* (Schum.:Fr.) P. Karsten; and (4) find possible connection between wood terpenoids (results published in detail in Manninen et al., 2002) and the measured performance parameters of a wood-boring insect and a decaying fungus.

METHODS AND MATERIALS

Growth Conditions and Sampling. In open pollinated stands of Scots pine (*Pinus sylvestris* L.), seeds were collected along a 1200-km north–south transect from seven geographical locations in Finland and two in Estonia. A total of nine provenances were used and ranged from Muonio (MUO) (latitude 67°56′), Ylitornio (YLI) (latitude 66°20′), and Suomussalmi (SUO) (latitude 65°10′) in the north, to Kinnula (KIN) (latitude 63°32′), Korpilahti (KOR) (latitude 62°0′), and Ruokolahti (RUO) (latitude 61°26′) in central Finland, and Tenhola (TEN) (latitude 60°3′), Rakvere (RAK) (latitude 59°18′), and Saaremaa (SAA) (latitude 58°22′) in the south. Seeds were sown in seedling beds at the Finnish Forest Research Institute

at Suonenjoki Research Station in spring 1991. In May 1993, 1000 seedlings from each of the nine provenances were planted at Suonenjoki Research Station in a nursery field that had been uncultivated for many years. The seedlings were randomly divided into five replicate blocks. Other details of growth conditions are described in Manninen et al. (2002).

In October 1998, 15 randomly selected 7-year-old trees from each provenance (three trees from each block) were cut. Internodes in the main stem were numbered from the terminal bud onwards. Fifth and sixth internodes with bark were sawed and stored in a paper bag at room temperature until processed for insect experiments. Wood samples (with bark or without bark, respectively) were collected from the fourth internode and stored at -20°C (decay tests) or -80°C (chemical analyses) for decay tests and chemical analyses.

Experiments with Hylotrupes bajulus. In culturing and rearing of adults and larvae of the old house borer *Hylotrupes bajulus* (Coleoptera: Cerambycidae), techniques described earlier were mainly utilized (Berry, 1972; European Standard EN 47, 1988). Larvae were mass-reared at the Institut für Holzbiologie und Holzschutz, Hamburg, Germany. Larvae were grown on protein–yeast-enriched pine sapwood at 27°C, 70% RH, and under constant dark to accelerate the growth of larvae (Berry, 1972; European Standard EN 47, 1988). In the fall of 2000, wood blocks with bark approximately 8-cm-long were sawed from the air-dried fifth annual growth from each tree (15 trees per provenance), from all nine provenances. A groove, $4.5 \times 1.5 \times 1$ cm was gouged on to one side of the wood blocks. They were individually weighed and, thereafter, one weighed larva (initial weight about 200–500 mg, total of 15 larvae per provenance) was enclosed into each groove under the glass plate. The wood blocks with glass plates were enclosed inside plastic boxes (size $14 \times 10 \times 4.5$ cm) having plastic lids with small breathing holes. During the experiment, boxes were kept in a dark culture room with air temperature approx. 21°C and R.H. about 60%. After 124 days, blocks were opened, and larvae were individually weighed. Mortality of larvae feeding on KIN, SUO, and MUO provenance wood was 6.7%; mortality of larvae feeding on RAK and RUO was 13.3%. All larvae feeding on wood from SAA, TEN, KOR, and YLI provenance stayed alive during the feeding period. The mean relative growth rate ($\mathrm{MRGR} = [\ln(w_2) - \ln(w_1)]/t$; w_2 = final mass, w_1 = initial mass, t = duration of the growth trial) for living larvae was calculated (depending on the provenance $N = 13$–15). The wood blocks were split, and frass was carefully collected from feeding galleries. Frass and remaining recombined wood blocks were separately weighed. The amount of wood consumed by each larva was determined by filling the empty feeding galleries with fine sand. Weight of sand corresponded to a certain volume, which was verified by several measurements. When the density of wood was known, the exact wood consumption by one larva could be determined.

In January 2001, four 7-mm-thick wood disks (diam. approximately 2–4 cm, depending on the provenance) were sawed from the air-dried sixth annual growth

of each tree ($N = 15$) belonging to SAA, TEN, KOR, SUO, and MUO provenances only (total of 360 disks). These provenances were selected on the basis of their variable terpenoid profile. Wood disks were used in 59 consecutive oviposition experiments of adult *H. bajulus* females. They were placed in random order on the outermost circles of 15-cm-diam. glass Petri dishes on the surface of dry filter paper. Thus, during every oviposition set, each Petri dish had six wood disks from the studied provenances. Adult *H. bajulus* males and females originated from the same rearing as above. One male and one female were allowed to copulate in a closed container in daylight for approx. one hr. Afterwards, mated females were released into the middle of a Petri dish containing wood disks, and allowed to lay eggs overnight in a dark culture room. The number of eggs laid under each wood disk was counted during the following day. Thereafter, mating was repeated to activate egg-laying. If eggs were found, females were offered other wood disks for further egg-laying tests. Experiments were conducted in the laboratory with an air temperature approximately 25°C and air RH of about 40%. A total of 29 different *H. bajulus* females were used in oviposition tests. Depending on the female, the total number of eggs produced was a product of 1–5 consecutive oviposition sets.

Decay Tests. A $30 \times 10 \times 5$ mm wood block from each tree without bark was cut. Decay resistance was studied at VTT Building and Transport using a malt agar plate decay test, which is a modification of the standardized EN 113 test (Viitanen et al., 1998; Venäläinen et al., 2001). The weighed, non-extracted wood sections were placed onto a pure culture of a brown-rot fungus, *Coniophora puteana*. The incubation time was 6 weeks, after which the samples were dried and re-weighed. Weight loss expressed as an absolute measure per fresh wood volume (mg/cm^3) was used as an inverse measure of decay resistance.

Chemical Analyses. Detailed results from the terpene and resin acid analyses of wood have already been published (Manninen et al., 2002). Wood samples for terpene analysis were stored in a deep-freezer (−80°C) until cut into small pieces and extracted with *n*-hexane using 1-chlorooctane as an internal standard (Manninen et al., 2002). Resin acids were extracted from freeze-dried and powdered wood samples according to Gref and Ericsson (1985). Heptadecanoic acid was used as an internal standard. Terpene and resin acid samples were analyzed with gas chromatography–mass spectrometry using a 30-m-long HP-5MS capillary column (Manninen et al., 2002). Individual terpenes and resin acids were identified by their mass spectra and retention times; concentrations were quantified by means of peak areas by using pure standards for calibration.

Statistical Analyses. Results of the performance parameters of *H. bajulus* larvae were split into two weight classes, small larvae between 100 and 300 mg and big larvae >300 mg, on the basis of initial larval fresh weight. Differences in the mean relative growth rate of both larval groups between provenances were analyzed with the covariance analyses (GLM univariate procedure) using initial larval fresh weight as a covariant. Other performance parameters of small larvae and

results of decay tests were analyzed using one-way analyses of variance (one-way ANOVA), and the provenance means were separated with the Tukey B procedure. Other performance parameters of big larvae that were not normally distributed after log-transformation and Kruskall–Wallis test and Mann–Whitney test with Bonferroni correction into P-values were used for that data. Oviposition results were coded female-specific, and the total number of eggs under each tree individual was calculated. Total egg number was normally distributed after log-transformation and ANOVA, and the Tukey B procedure in separation of provenance means was used. Correlations were determined using non-parametric Spearman correlation. Hierarchical cluster analyses from the between-provenances squared Euclidean distance were used to separate the provenances on the basis of stem wood chemistry. All analyses were done with SPSS for Windows 10.0 statistical software.

RESULTS

Performance of H. bajulus. In the combined data, the mortality of *H. bajulus* larvae feeding on air-dried Scots pine wood during the 124 days was 5.2%. Small larvae ($N_{small} = 52$) (initial weight 100–300 mg) lost less weight during the experiment than big larvae ($N_{big} = 62$) (initial weight >300 mg), and had a higher mean relative growth rate (MRGR) except on SUO and YLI provenances (Figure 1). MRGR of big larvae was negative in all the provenances and was not significantly ($F = 1.443$, $P = 0.201$, df = 8) different among provenances (Figure 1). Also,

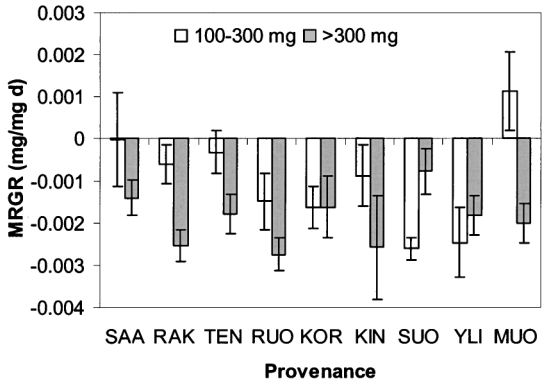

FIG. 1. The mean relative growth rate (MRGR±SE) of small (initial larval weight 100–300 mg, $N_{small} = 52$) and big (initial larval weight >300 mg, $N_{big} = 62$) *Hylotrupes bajulus* larvae during 124 days feeding on Scots pine wood from nine different provenance. Provenance names: SAA = Saaremaa, RAK = Rakvere, TEN = Tenhola, RUO = Ruokolahti, KOR = Korpilahti, KIN = Kinnula, SUO = Suomussalmi, YLI = Ylitornio, and MUO = Muonio.

the MRGR of small larvae was negative in all the other provenances, except in MUO provenance (Figure 1). The MRGR of small larvae was marginally higher ($F = 2.167$, $P = 0.050$, df = 8) on wood from MUO than on wood from either of the other two northern provenances, SUO and YLI (Figure 1). However, on the basis of individual resin acid concentrations in the stem wood (Table 1), MUO and YLI provenance belonged in the same cluster and SUO in another cluster dominated by more southern provenances other than SAA and KOR (Figure 2). Table 1 shows that all individual resin acid concentrations in wood from YLI and MUO provenances were lower than in wood from the other provenances. The MRGR of small larvae positively correlated with the total monoterpene concentration in the stem wood ($r = 0.339$, $P = 0.014$), but this was not detected with the big larvae. If the MRGR of both small and big larvae are pooled, correlation with wood monoterpenes becomes very weak ($r = 0.190$, $P = 0.043$), and MRGR does not correlate with wood resin acids at all ($r = 0.156$, $P > 0.05$).

Small and big larvae consumed about 1.4 and 1.8 g of air-dried wood, respectively, during the 124-day feeding period. Provenance affected the amount of consumed wood by small larvae (one-way, $P = 0.045$), but not the amount of frass produced. They consumed most wood on YLI provenance and least wood on SUO and KIN provenance (Table 2). However, consumption did not differ significantly from each other in Tukey's test. Feeding was higher on wood that contained a higher proportion of levopimaric+palustric acid (Spearman correlation coefficient 0.349, $P = 0.011$, data not shown) and lower proportion of abietic acid ($r = -0.290$, $P = 0.037$, data not shown). On the other hand, provenance affected both the amount of consumed wood by big larvae (Kruskal–Wallis, $P = 0.003$) and the amount of frass produced (Kruskal–Wallis, $P = 0.033$). Larvae consumed more wood and produced more frass (Table 2) when feeding on wood from YLI provenance than on wood from RAK (Mann–Whitney, $P = 0.036$) or RUO (Mann–Whitney, $P = 0.036$) provenances. YLI provenance had lower resin acid concentrations in the stem wood than RAK and RUO (Table 1), and on that basis belonged in a different cluster (Figure 2).

Oviposition of H. bajulus. The total number of eggs laid by 29 *H. bajulus* adult females in 59 consecutive experiments was 3990, and their distribution is presented in Table 3. Provenance affected the amount of eggs (one-way, $P = 0.022$), and the mean number of eggs was significantly (Tukey, $P < 0.05$) higher under wood disks originating from the northernmost MUO provenance than under the disks from SAA, TEN, or SUO provenances (Figure 3). Only TEN, and SUO provenances belonged in the same cluster on the basis of individual resin acid concentration in the stem wood (Figure 2). Table 1 shows that wood from SAA provenance contained distinctly higher resin acid concentrations than TEN and SUO provenances. If a similar dendrogram were produced on the basis of individual monoterpene concentration in stem wood (data not shown), KOR, YLI, and MUO provenances would form one cluster, and SAA would be left in its own cluster.

TABLE 1. MEAN CONCENTRATION (SE IN PARENTHESES) OF INDIVIDUAL AND TOTAL MONOTERPENES (μg g^{-1} f.wt.) AND RESIN ACIDS (mg g^{-1} d.wt.) IN THE WOOD OF SCOTS PINE TREES REPRESENTING DIFFERENT SEED PROVENANCES[a]

Compound group and compound	Provenance								
	SAA South	RAK	TEN	RUO	KOR	KIN	SUO	YLI	MUO North
Monoterpenes									
Tricyclene	tr	tr	tr	tr	tr	tr	tr	tr	tr
α-Pinene	248.5 (30.1)	277.3 (25.8)	218.2 (16.7)	246.4 (34.3)	180.9 (23.7)	276.1 (26.0)	238.1 (28.5)	221.3 (23.9)	272.5 (27.0)
Camphene	4.0 (0.6)	5.6 (0.7)	3.7 (0.4)	5.0 (0.9)	3.4 (0.7)	5.2 (0.6)	4.7 (0.8)	4.2 (0.6)	5.8 (0.7)
Sabinene	29.8 (1.8)	16.9 (3.5)	20.2 (2.4)	14.7 (2.9)	9.1 (1.7)	16.5 (2.6)	14.1 (2.3)	8.4 (2.2)	7.9 (2.0)
β-Pinene	24.6 (6.2)	35.9 (7.3)	20.1 (3.5)	17.5 (3.5)	12.0 (2.0)	26.4 (7.6)	17.4 (4.4)	18.5 (3.8)	28.1 (10.8)
Myrcene	25.7 (1.5)	27.4 (3.1)	23.3 (2.4)	22.4 (2.3)	16.2 (1.6)	22.2 (2.7)	20.7 (2.1)	18.2 (2.0)	18.9 (2.1)
3-Carene	398.8 (20.8)	193.0 (41.3)	284.9 (26.3)	196.0 (38.9)	136.8 (25.0)	228.9 (31.8)	188.4 (33.1)	115.2 (32.7)	104.8 (30.9)
Limonene[b]	40.6 (17.3)	55.2 (15.8)	36.4 (9.2)	74.7 (11.3)	50.5 (8.4)	62.8 (5.8)	67.9 (10.3)	72.5 (15.6)	87.0 (15.2)
Terpinolene	63.1 (4.5)	33.9 (7.0)	41.4 (4.7)	29.2 (5.7)	18.5 (3.4)	33.4 (4.9)	28.2 (4.9)	16.3 (4.2)	16.4 (4.1)
Bornylacetate	tr	tr	tr	tr	tr	1 (0)	tr	tr	1 (0)
Total monoterpenes	837.1 (59.3)	646.9 (40.5)	649.8 (39.4)	607.8 (40.0)	428.6 (27.7)	674.1 (27.8)	580.8 (33.0)	475.9 (20.0)	544.4 (25.5)
Resin acids									
Pimaric	0.80 (0.07)	0.53 (0.05)	0.47 (0.05)	0.64 (0.10)	0.76 (0.11)	0.60 (0.06)	0.54 (0.06)	0.48 (0.06)	0.43 (0.04)
Sandaracopimaric	0.32 (0.03)	0.21 (0.02)	0.21 (0.01)	0.23 (0.02)	0.30 (0.04)	0.24 (0.02)	0.21 (0.02)	0.19 (0.02)	0.17 (0.01)
Isopimaric	0.25 (0.04)	0.19 (0.04)	0.17 (0.03)	0.15 (0.03)	0.18 (0.03)	0.17 (0.05)	0.15 (0.03)	0.12 (0.02)	0.15 (0.03)
Palustric+Levopimaric	8.22 (0.70)	5.46 (0.42)	5.53 (0.41)	6.20 (0.72)	8.02 (1.17)	6.35 (0.50)	5.22 (0.36)	5.08 (0.73)	4.35 (0.36)
Dehydroabietic	0.49 (0.04)	0.44 (0.05)	0.46 (0.05)	0.40 (0.03)	0.47 (0.05)	0.46 (0.05)	0.39 (0.03)	0.39 (0.04)	0.31 (0.03)
Abietic	10.13 (1.20)	6.07 (0.67)	6.24 (0.49)	7.01 (0.83)	8.73 (1.10)	7.91 (0.73)	6.69 (0.89)	5.06 (0.79)	4.02 (0.36)
Neoabietic	2.62 (0.21)	1.81 (0.14)	1.64 (0.13)	1.93 (0.21)	2.78 (0.44)	2.12 (0.17)	1.73 (0.12)	1.49 (0.17)	1.49 (0.12)
Total resin acids	22.83 (2.15)	14.71 (1.26)	14.72 (0.98)	16.56 (1.88)	21.24 (2.84)	17.84 (1.39)	14.93 (1.33)	12.81 (1.76)	10.92 (0.81)

[a] SAA = Saaremaa, RAK = Rakvere, TEN = Tenhola, RUO = Ruokolahti, KOR = Korpilahti, KIN = Kinnula, SUO = Suomussalmi, YLI = Ylitornio, MUO = Muonio ($N = 15$). The provenances are ordered according their geographical distribution.

[b] Includes also β-phellandrene, because limonene and β-phellandrene were not separated from each other on HP-5MS column. tr, trace compound (concentration <1 μg g^{-1} f.wt.).

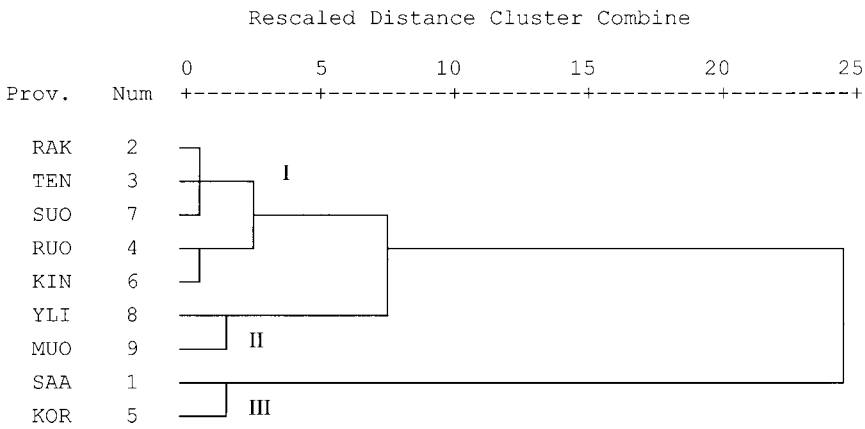

FIG. 2. Dendrogram based on the mean individual resin acid concentrations in wood. Provenance names as described in Figure 1. Cluster method: between groups linkage, interval measure: squared euclidean distance. Three clusters are numbered with Roman numerals.

About half of the eggs were laid under the wood disks originating from KOR and MUO provenances; about 20% of eggs were laid under wood disks from KIN provenance (Table 3). In 44 out of 59 experiments, females laid their eggs under the wood disk of only one provenance. However, in 11 experiments, eggs were found under the disks of two different provenances, and in 5 experiments under

TABLE 2. WOOD BIOMASS CONSUMED AND FRASS PRODUCED (SE IN PARENTHESES) BY SMALL (INITIAL LARVAL WEIGHT 100–300 mg) OR BIG (INITIAL LARVAL WEIGHT >300 mg) *Hylotrupes bajulus* LARVAE DURING 124 DAYS FEEDING ON AIR-DRIED SCOTS PINE WOOD FROM NINE DIFFERENT PROVENANCE

	Wood biomass consumed (g)		Frass produced (g)	
Provenance	Small larvae	Big larvae	Small larvae	Big larvae
Saaremaa	1.56 (0.29)	1.82 (0.16)	2.08 (0.28)	2.44 (0.14)
Rakvere	1.37 (0.10)	1.27 (0.14)b	1.67 (0.09)	1.71 (0.15)b
Tenhola	1.65 (0.19)	1.58 (0.22)	1.69 (0.26)	2.16 (0.31)
Ruokolahti	1.43 (0.04)	1.22 (0.28)b	1.61 (0.11)	1.68 (0.39)b
Korpilahti	1.57 (0.18)	2.06 (0.30)	1.56 (0.14)	2.56 (0.37)
Kinnula	1.20 (0.13)	1.26 (0.26)	1.58 (0.17)	1.88 (0.33)
Suomussalmi	1.02 (0.04)	1.99 (0.18)	1.48 (0.11)	2.51 (0.24)
Ylitornio	1.78 (0.14)	2.57 (0.11)a	1.79 (0.20)	3.03 (0.23)a
Muonio	1.33 (0.12)	1.70 (0.20)	1.63 (0.05)	2.18 (0.32)

Note. In the combined data $N_{small} = 52$, $N_{big} = 62$. Means among weight class followed by different letters are significantly different according to Mann–Whitney U test.

TABLE 3. DETAILS OF THE OVIPOSITION EXPERIMENT WITH *H. bajulus* FEMALES

Provenance	Egg-laying occasions[a]	Number of females[b]	Total number of eggs laid	Eggs (%) from total amount	Number of trees selected (N max. 15)
Saaremaa	11	8	463	11.6	7
Tenhola	9	8	312	7.8	7
Korpilahti	18	17	1040	26.1	11
Kinnula	13	8	788	19.7	10
Suomussalmi	10	9	377	9.4	7
Muonio	19	18	1010	25.3	8

[a] How many times at least one egg was laid under the provenance.
[b] The number of individual females out of 29, which selected the provenance at least once.

the disks of three different provenances. Only seven females were devoted solely to one provenance during the whole egg-laying experiment. More than half of individual females selected wood disks from KOR or MUO provenance at least once as an oviposition site; approximately 30% of females also selected wood from other provenances as an oviposition site (Table 3).

Not all individual trees were suitable as an oviposition site for *H. bajulus*, since wood disks from 40 trees out of 90 possible were not selected once. From KOR and KIN provenance, 12 and 10 individual trees out of 15 received eggs, respectively (Table 3). In all the other provenances, about half of the trees were not selected as an oviposition site, although they were offered as often as the other trees (Table 3). The total monoterpene, ($r = -0.288$, $P = 0.043$) and β-pinene ($r = -0.409$, $P = 0.003$) concentrations, and $\beta : \alpha$-pinene ratio ($r = -0.496$, $P < 0.001$) in

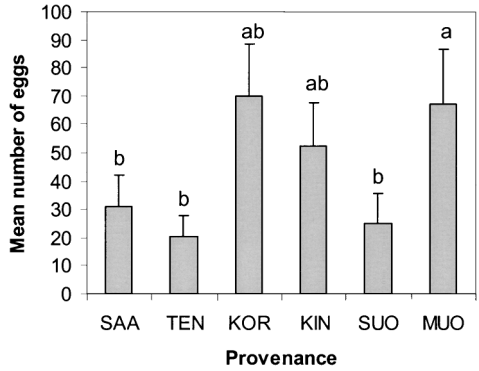

FIG. 3. Mean number of eggs laid (+SE) by adult *Hylotrupes bajulus* females under the Scots pine wood disks originating from six different provenances. Provenance names as described in Figure 1. Means followed by different letters are significantly different according to Tukey B.

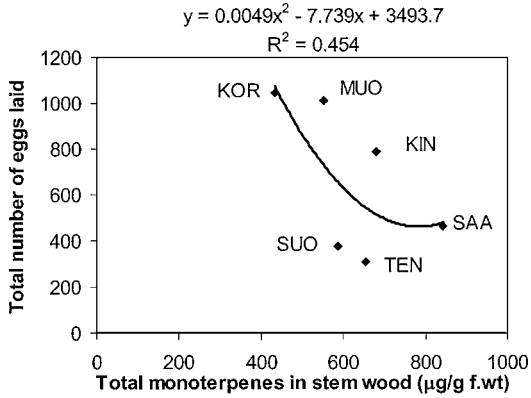

FIG. 4. Polynomial relationship between the total monoterpene concentration in the wood of Scots pines representing six different provenance and the total number of eggs laid by adult *Hylotrupes bajulus* females. Provenance names as described in Figure 1. Equation for relationship and r^2 are given.

the wood negatively correlated with the total number of eggs. Actually, the relationship between mean total monoterpene concentration in the stem wood of each provenance and the total number of laid eggs followed a polynomial line (Figure 4).

Decay Resistance. The mass loss of wood per fresh wood volume in Figure 5 describes the degrading activity of the fungus. Significant ($P = 0.009$) differences between Scots pine provenances in the durability of wood against the brown rot fungus *C. puteana* were found (Figure 5). Wood from SAA, TEN, RUO, and SUO provenance was more resistant to decay than wood from KIN provenance

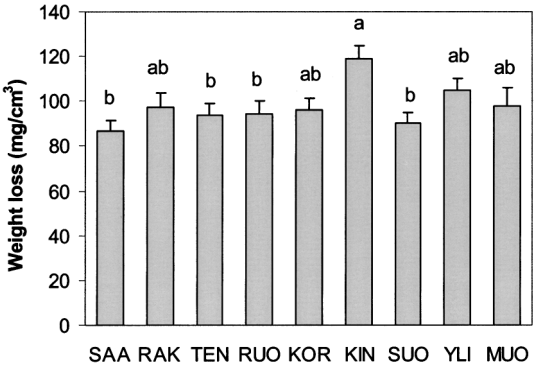

FIG. 5. The weight loss (+SE) of wood representing nine different Scots pine provenance in decay test with *Coniophora puteana*. Provenance names as described in Figure 1. Means followed by different letters are significantly different according to Tukey B. $N = 15$.

(Figure 5). Terpene and resin acid concentrations in the wood did not clearly correlate with decay resistance of the same wood material (data not shown). However, the total wood resin acid concentration of an individual tree negatively correlated with the decay resistance of wood in some provenances, i.e., SAA ($r = -0.622$, $P = 0.013$, $N = 15$) and KOR ($r = -0.579$, $P = 0.024$, $N = 15$), and total monoterpene concentration of wood negatively correlated with the decay resistance of RAK ($r = -0.664$, $P = 0.007$, $N = 15$) and KIN ($r = -0.593$, $P = 0.020$, $N = 15$) provenance wood. Cluster analyses failed to combine more decay-resistant provenances into the same cluster on the basis of individual resin acid concentration in wood (Figure 2), mainly because of the high resin acid concentrations found in wood from SAA provenance (Table 1). Also, wood terpene concentrations did not cluster all four provenances (data not shown), because wood from SAA contained more terpenes than the other provenances (Table 1 and see Manninen et al., 2002).

DISCUSSION

This research demonstrates that individual monoterpenes or resin acids from wood of different Scots pine provenances better explain the oviposition choice of *H. bajulus* females than total concentration. Larval performance, however, is related to higher total monoterpene content or associated with a high proportion of certain resin acids in wood. However, terpenoids do not explain the decay resistance of Scots pine juvenile wood.

Rasmussen (1961) suggested that *H. bajulus* larvae exhibit a linear growth curve up to 200 mg. However, we divided larvae into two equal-size groups and used larval initial weight of 300 mg as cutoff weight. In general, small larvae were more responsive than big larvae. The mean relative growth rate for *H. bajulus* larvae during 124 days feeding was mostly negative regardless of the weight class. One reason for this might be the high fat content of larvae pregrown on nutrient-rich pinewood to accelerate growth (Berry, 1972). When transferred to normal air-dried Scots pine wood with low nutritional quality, larvae continued their feeding probably only to the extent that daily metabolic needs were maintained, and they lost weight. Rasmussen (1961) reported that weight loss was directly related to fat reserves of larvae. Growth of *H. bajulus* is most rapid at 28–30°C with an optimum RH of 80–90% (Robinson and Cannon, 1979; European Standard EN 47, 1988). During the current experiment, both temperature and RH were below the optimum, which could have slowed larval growth. Also, wood moisture content could have affected the feeding habits of larvae. Cannon and Robinson (1981) did not find differences in wood consumption and growth of *H. bajulus* when grown near optimum or below optimum conditions; however, consumption and growth were decreased in the attics, where the environmental conditions (temperature, RH, and wood moisture content) fluctuate.

H. bajulus larvae live in dry and seasoned sapwood with low nutritional quality. The resistance of wood against this insect is dependent upon tree species. Pine has been found to be more susceptible to *H. bajulus* damage than either spruce or fir (Graf et al., 1989). The most important nutrient component for development of larvae is the protein content of wood species (Holm and Ekbom, 1958; Graf et al., 1989). Along with protein, larvae can utilize cellulose and hemicellulose by using cellulose-digesting enzymes (Hanks, 1999). The protein and nutrient content of wood from different provenances was not analyzed in this study. However, if construction timber has been produced from trees with low vitality, i.e., low needle volume and low photosynthesis, and such timber might have lower nutrient quality and increased resistance to *H. bajulus* damage (Graf et al., 1989). Slow-growing trees of northern provenances have proven to be the most highly defended against herbivore pressure, with respect to lignin content of Sitka spruce bark (Wainhouse and Ashburner, 1996) and monoterpene content of Scots pine needles (Manninen et al., 1998) or Sitka spruce terminals (Hrutfiord and Gara, 1989). Results of the present work concerning terpenoid-based defense of wood do not support this, because the most northern Muonio provenance had the lowest resin acid concentration in wood (Manninen et al., 2002). Also, it should be noted, that wood production of Scots pine has increased when transferred southwards where annual mean temperature is higher (Beuker, 1994).

There are not many studies where the role of conifer terpenoids has been specified in determining the performance of wood-boring insects (Holm and Ekbom, 1958; Fettköther et al., 2000). There are no data available that show how terpenes affect the growth rate of *H. bajulus* larvae. In general, orientation of *H. bajulus* females is guided by light and by odors of monoterpenes ($C_{10}H_{16}$), especially α-pinene (Mares et al., 1986; Fettköther et al., 2000). β-Pinene activates *H. bajulus* females, but it does not orient them towards the scent source (Fettköther et al., 2000). Wood terpenes do affect the selection of oviposition place of *H. bajulus* and the activity of adults (Holm and Ekbom, 1958; Fettköther et al., 2000). In our study, better MRGR of small larvae was associated with a higher concentration of wood monoterpenes. In addition, small *H. bajulus* larvae had better MRGR on wood that had the lowest total resin acid concentration (MUO provenance). The lowest MRGR occurred on wood from SUO and YLI provenances, but their resin acid concentration did not differ from that of MUO. Actually, YLI and MUO provenances were clustered on the basis of stem wood resin acid concentration. Contrasting with the study of Mares et al. (1986), α-pinene alone did not explain the oviposition preference of *H. bajulus* females in our study, but β-pinene concentration negatively correlated with the number of eggs. Because a low $\beta : \alpha$-pinene ratio of stem wood best explained the high number of laid eggs and total monoterpenes negatively correlated with the number of eggs, our results suggest that odors of individual monoterpenes, and especially their ratios, might be more important in determining oviposition behavior of *H. bajulus* than total monoterpenes.

A polynomial relationship between the stem wood monoterpenes and total number of eggs might suggest that intermediate monoterpene concentration is not attractive for ovipositing adults.

The composition of oleoresin compounds is genetically controlled (Hiltunen et al., 1975; Gref, 1987; Baradat and Yazdani, 1988), which probably has had a strong effect on the oleoresin chemistry of stem wood in the provenances studied. Transfer of Scots pine provenances from their original latitudes has been shown to change terpene, resin acid, and total phenolics concentration in the needles (Nerg et al., 1994). In addition, Ståhl (1998) has reported that Scots pine provenances transferred southwards will form thin annual rings, high basic density, and lower diameter tracheids with thick cell walls in comparison to local provenances. These changes in wood density and length of tracheids could affect the ability of *H. bajulus* larvae to bite and digest wood material. Unfortunately, the anatomical properties of stem wood were not analyzed in this study. Therefore, it might not be possible to use current results to evaluate the importance of stem wood chemistry in *H. bajulus* performance at the original latitudes of the provenances.

Cannon and Robinson (1981) found that larvae belonging to different weight classes consumed nearly the same amount of wood during the experiment (90 days). In our experiment, the amount of consumed wood during 124 days feeding was 22% higher among big larvae than among small larvae. It appeared that wood consumption of small larvae was not associated with the provenance at all, but wood consumption was associated with the proportional quantities of certain resin acids in the stem wood. Big larvae consumed (i.e., excavated) most wood from YLI provenance, and produced the highest amounts of frass. However, these larvae did not have more rapid development or higher MRGR than larvae grown on wood from the other provenances. It might be possible that wood from YLI provenance has shorter tracheids and, thus, less cellulose, leading to reduced nutritive value for the larvae. In our other experiment, the MRGR of *H. bajulus* larvae was better on Scots pine wood having long tracheids (Heijari et al., unpublished). In general, wood material used in our study was juvenile wood, which has shorter tracheids than mature wood.

The decay resistance of Scots pine wood was highest in wood blocks originating from SAA, TEN, and SUO provenances, but cluster analysis showed a long distance of SAA provenance from TEN and SUO provenance on the basis of wood resin acid concentrations. Interestingly, the same three provenances had the lowest number of eggs laid by *H. bajulus*. In addition, wood from RUO provenance was more resistant to decay than wood from KIN provenance. Several resin acids inhibit the fungal growth of wood-rotting fungi (Henriks et al., 1979; Eberhardt et al., 1994; Micales et al., 1994), and decay-resistant Scots pine trees contain more phenolics and resin acids in heartwood than decay-susceptible trees (Harju et al., 2002; Venäläinen et al., 2003). It is also known that heartwood is more resistant to

decay than sapwood (Hart and Shrimpton, 1979). In the present study, heartwood was not yet developed in the young saplings. Thus, the wood contained mostly monoterpenes and resin acids, but probably not phenolics. Overall, the role of resin acids in the decay resistance of natural wood substrate has been interpreted to be minor compared to that of phenolics (Venäläinen et al., 2003). One reason for the difference in decay resistance of different provenances could be changes in the cellulose and/or lignin concentrations of wood. *C. puteana* is a cellulose decomposing fungus, and either environmental factors or genetic traits may cause changes in cellulose and/or lignin concentration, which could affect the substrate available for the fungus (Harju et al., 2001). Lignin content of Sitka spruce bark is genetically controlled, but also strongly influenced by environmental factors (Wainhouse and Ashburner, 1996). It is also possible that transfer of provenances may interact with the genetic determination of decay resistance, as suggested by Venäläinen et al. (2001).

In summary, wood from SAA, TEN, and SUO provenances had the fewest eggs of *H. bajulus* and were most resistant against decay fungi. MRGR of small larvae on wood from YLI provenance was lowest. Chemical analyses of wood terpenoids did not explain all the observed differences. For example, total monoterpene concentration in wood had opposite effects on MRGR of larvae and oviposition of adults. It also appears that individual terpenoid compounds are more important in determining the performance of wood-boring insects than total amounts. Further study needs to determine whether structural properties of wood, nutritive wood value, and cellulose and lignin content play significant roles in insect and fungal deterrence.

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RESISTANCE OF NEONATES AND FIELD-COLLECTED GARTER SNAKES (*Thamnophis spp.*) TO TETRODOTOXIN

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Abstract—Prior studies of tetrodotoxin (TTX) resistance in garter snakes (*Thamnophis spp.*) have used laboratory-reared neonates as subjects, but the use of field-caught individuals would reduce cost and effort. We compared estimates of TTX resistance in field-caught and laboratory-born garter snakes. We found that a mass-adjusted dose of TTX administered to field-caught garter snakes produces an estimate of a population 50% dose that is comparable and unbiased with respect to those previously reported using laboratory-born neonates. Dose-response curves estimated for three field-caught populations closely matched the curves estimated from neonate data. The method was tested using populations with levels of TTX resistance ranging between approximately 5–90 mass-adjusted mouse units for their respective 50% doses. The technique of using field-caught snakes as test subjects provides larger genetically independent data sets that are more easily obtained. Our results indicate that changes in mass during development parallel ontogenetic shifts in TTX resistance.

Key Words—Tetrodotoxin, resistance, dose-response curve, mass adjustment, *Thamnophis sirtalis*, *Thamnophis couchii*.

INTRODUCTION

The study of drug resistance and toxicity is important to disciplines that range from biomedical research to agriculture. Given this broad range, it is not surprising that bioassays measuring resistance or toxicity are usually tailored to the system and question of interest. Many newer techniques have arisen as improved alternatives to prior methods. The improvement may come in the form of reduced cost, higher

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throughput, reduced effort, or alternatives to laboratory animal usage (see Raposo et al., 1995; Plasencia and Banttari, 1997; Spielmann et al., 1999; Sousa and Poiares da Silva, 1999; Olmstead et al., 2001; Valentin-Severin et al., 2002, for examples). Although there are many ways to measure resistance or toxicity, two categories of assays are particularly prevalent. The first method, referred to as the 50% lethal dose or LD_{50}, measures the dose that is lethal to 50% of the test organisms (Kadir et al., 1999; Maa and Liao, 2000; Schmuck et al., 2001). The second, called the 50% inhibition concentration or IC_{50}, measures the dose at which a biological function (e.g., growth or binding rate) is inhibited by 50% (Sousa and Poiares da Silva, 1999; Lee and Adams, 2000; Ismail et al., 2002). The use of measures such as the LD_{50} or IC_{50} provides a simple way to compare the potency of a toxin or resistance across toxins or organisms. However, LD_{50} and IC_{50} only measure the *average* response of individuals within a population and are not useful for assessing individual variation. The estimation of *individual* differences in trait values is fundamental to the study of evolution.

The coevolutionary interaction between the common garter snake *Thamnophis sirtalis* and the rough-skinned newt *Taricha granulosa* revolves around tetrodotoxin (TTX) toxicity and resistance. *Taricha granulosa* (and other newts of the genus *Taricha* found in western North America) possess TTX as a potent chemical defense (Brodie, 1968). In an apparent coevolutionary arms race, the common garter snake *Thamnophis sirtalis* has evolved TTX resistance in parallel with the toxicity level of *Taricha granulosa* (Brodie et al., 2002). By measuring the ratio of unimpaired crawl speed to TTX impaired crawl speed of neonate garter snakes, Brodie and Brodie (1990) developed an assay that estimates individual variation in resistance. The geographic mosaic theory of coevolution (Thompson, 1994) predicts that due to evolutionary forces other than selection, populations within the larger metapopulation will have different evolutionary endpoints (i.e., there will be variation among populations for trait values). Therefore, Brodie et al. (2002) developed a population resistance measure similar to an IC_{50} to allow for easy comparison among groups; the so-called 50% TTX dose is obtained by regression techniques utilizing the data from the individual assay of Brodie and Brodie (1990). In combination, the methods of Brodie and Brodie (1990) and Brodie et al. (2002) provide a technique that yields both the individual measures of TTX resistance necessary to address evolutionary questions and also a measure similar to an IC_{50} that is useful for comparison across groups.

The TTX resistance bioassay (Brodie and Brodie, 1990) used neonate garter snakes as test subjects. The use of neonates is advantageous for several reasons. First, little is known about the ontogenetic change in TTX resistance levels in *T. sirtalis* other than that TTX resistance cannot be altered through exposure (Ridenhour et al., 1999). Using neonates provides a set developmental time point at which testing may occur and be compared across populations (approximately 5-days postbirth). Second, the use of neonates provides a distribution of resistance

within the population prior to selective influences. Third, garter snakes' litters can be large; one gravid female can provide multiple data points. The use of neonates, however, has problems. Capture of gravid female *T. sirtalis* can be difficult due to the reduced number of potential animals in the target population; capture of gravid females from small populations is particularly difficult. Once gravid females are caught, they must be returned to the laboratory and cared for. Husbandry of gravid females is expensive, time-consuming, and may produce no offspring in the end. Finally, although litters produce larger sample sizes, they are not genetically independent data points and, thus, overestimate the confidence placed in the resistance distribution.

We tested an alternative method of measuring TTX resistance in garter snakes by comparing population estimates of resistance in field-caught *T. sirtalis* and *T. couchii* to neonate assays from the same populations. The use of field-caught garter snakes obviates the problems associated with the capture and husbandry of gravid females and, given that collected individuals are not littermates or from highly inbred populations, provides genetically independent resistance measures. Field-caught animals, both male and female, were captured from populations with known levels of neonate resistance and assayed for resistance by using a mass-adjusted TTX dose. The estimated 50% dose from the field-caught snakes was then compared to the estimated 50% dose, using neonate snakes.

METHODS AND MATERIALS

Resistance of neonate garter snakes was estimated in two species. Gravid female *T. sirtalis* were collected from two different populations: Bear Ridge, Humboldt Co., CA ("Bear Ridge") and Adair, Benton Co., OR ("Benton"). Gravid female *T. couchii* were collected from Cold Springs, Tulare Co., CA ("Cold Springs"). Gravid females were housed individually in $25 \times 50 \times 30$ cm aquaria placed on heat-tape to generate a thermal gradient; each aquarium contained a sphagnum-filled hide box and a water bowl. Females were fed fish once per week. The animal chamber was held on a 12L:12D cycle at $26 \pm 1^{\circ}$C. Females were checked multiple times per day for the presence of neonates in their aquaria. Neonate snakes were housed separately in plastic tubs (15 cm diam. $\times$ 10.5 cm tall) and assayed for resistance starting approximately 3–5 days postparturition. Neonates were given water on a daily basis; on trial days, they were given water posttesting.

From each of the three populations, captured animals that were not gravid females were returned to the laboratory for resistance tests ("field-caught"). Field-caught animals were housed individually and kept in conditions identical to those of the gravid females. Field-caught individuals ranged from young-of-the-year ($\sim$4–7 g) to full-grown adults ($\sim$50 g). Assays for resistance on field-caught individuals were not performed at a predetermined time interval after entering captivity

(i.e., the time interval from capture to trial varied among individuals). All field-caught animals were considered to be in good condition prior to use in the experiment.

Using the methodology of Brodie and Brodie (1990), snakes were assayed for resistance to TTX. An adjustable electronic racetrack, 4 m in length, was used to measure crawl speeds. Because the field-caught snakes were larger than neonates, the racetrack was configured differently for running neonate and field-caught animals. For field-caught animals, the track was set to 20 cm in width; for neonates, it was set at 11 cm. Crawl speed for both categories was measured over the central 2 m of the track, with electronic sensors every 0.5 m (thus providing four half-meter speeds); the fastest 0.5 m segment was used as the maximum crawl speed of the snake. Animals were stimulated to crawl down the length of the track by tapping their tail with a finger. The baseline crawl speed for an animal was calculated as the average crawl speed from two separate uninjected time trials performed on the same day approximately 4 hrs apart. An artificial turf substrate was used to aid in crawling. All trials were performed at $26 \pm 1^{\circ}$C.

Garter snakes were assessed for intoxicated crawl speed 30 min after an i.p. injection. Individual snakes were repeatedly injected to test resistance at different doses, but no snake was injected more than five times *in toto*. Snakes were not injected on consecutive days to ensure the elimination of residual TTX effects (Brodie and Brodie, 1990) and to reduce the effect of fatigue. Resistance was measured as the ratio of crawl speed before injection to crawl speed after injection of TTX. For example, a resistance score of one indicates that a snake was unaffected by a given dose of TTX, while a score of 0.25 (i.e., 25% resistant) indicates that the snake's crawl speed was reduced by 75% at that dose.

The effective dosages were calculated in the following manner. A variable volume (up to 0.5 ml) of TTX solution of known concentration (mg TTX/ml amphibian Ringer solution) was administered via intraperitoneal injection. The absolute dose (mg TTX) was then transformed to a mass-adjusted mouse unit (MAMU). A mouse unit is the amount of TTX (0.0002857 mg) required to kill a 20-g mouse in 10 min. Mass-adjusted mouse units were calculated using different methods for field-caught and neonate snakes. For neonates, MAMU dose was calculated as

$$\text{dose}_{\text{n}} = \frac{\text{dose mg TTX}}{\bar{m}\,\text{g}} \times \frac{20\,\text{g}}{0.0002857\,\text{mg TTX}}$$

where $\bar{m}$ is the average mass of a neonate snake in the population of origin. The MAMU dose for field-caught animals was calculated as

$$\text{dose}_{\text{f}} = \frac{\text{dose mg TTX}}{m\,\text{g}} \times \frac{20\,\text{g}}{0.0002857\,\text{mg TTX}}$$

where m is the mass of the individual snake tested. The only difference between the two methods of calculating the dose is the use of the population mean mass, $\bar{m}$, in neonates vs. individual mass, m. The use of $\bar{m}$ also implies that neonates

were given the identical absolute amount of TTX (mg TTX) at a given MAMU dose. In contrast, field-caught individuals received variable absolute amounts of TTX for a given MAMU dose. Both measures adjust dose relative to mass and interpret this adjustment in the biologically relevant mouse unit. Because of the relatively low variance in mass at birth in a population of garter snakes, using $\bar{m}$ is effectively similar to using m in the equation but captures the expectation for an average neonate in the population.

The doses administered to snakes from the three populations varied. The use of different doses between populations was necessary due to the variability in population resistance levels. For example, the doses given to Bear Ridge snakes would have little to no effect on snakes from Benton or Cold Springs. The approximate range of doses given to field-caught animals was 5–100 MAMU for Benton, 2–17 MAMU for Bear Ridge, and 8–206 MAMU for Cold Springs. The approximate range of doses given to neonates was 12–253 MAMU for Benton, 3–19 MAMU for Bear Ridge, and 19–97 MAMU for Cold Springs. Doses were chosen for field-caught individuals on the basis of the 50% doses found in neonates (Brodie et al., 2002), and to be roughly equivalent to the doses given to neonates. The estimates for the 50% doses were 34.1 MAMU for Benton and 6.6 MAMU for Bear Ridge; no estimate has been published for Cold Springs neonates.

Data analysis was conducted by using SAS (version 8.0, SAS Institute, Inc.). To characterize the resistance level of neonate and field-caught animals, a 50% dose was calculated using curvilinear regression on natural-log-transformed dosages to provide a simple estimate of a population-wide 50% dose (Brodie et al., 2002). The curvilinear regression was performed by utilizing the linear regression $y' = \alpha + \beta x'$, where $y' = \ln(1/y - 1)$ and $x' = \ln(x)$; y is TTX resistance, x is TTX dose, and α and β are the estimated parameters. Data adjustment was performed in a manner slightly different from the method described in Brodie et al. (2002); data points that were greater than or equal to one were treated as 0.999, and those that were zero were treated as 0.001. The estimated 50% dose of field-caught animals was then compared to that of laboratory-born neonates to determine differences. Because the estimated 50% doses are ratios of two parameters, bootstrapping was done to create a sample t-distribution for the difference of two 50% estimates. The test statistic was chosen to test the hypotheses

H_0: $|\tau_n - \tau_f - \delta| > 0$ or H_1: $|\tau_n - \tau_f - \delta| = 0$, where τ_n and τ_f are the estimated 50% dose for neonates and field-caught animals, respectively. Because we sought to test equivalence of methods, H_1 tests the hypothesis of equality (i.e., a reverse test). A standard statistical test would only show that τ_n and τ_f were not equal and not truly test that the parameters were equal (the case in which we are interested). With the test we performed, the parameter δ estimates the observed difference between the two 50% estimates and produces equality in our hypothesis. Using a data set created from 1000 bootstrap samples, values of δ were found that satisfied H_1 at the $P < 0.05$ level (one-tailed), thus producing a range of the observed difference between the neonate and field-caught 50% doses.

TABLE 1. REGRESSION RESULTS FOR FIELD AND LABORATORY (NEONATE) GROUPS FROM THREE DIFFERENT POPULATIONS OF *Thamnophis.*

Population	N	N_i	α	P_α	β	P_β
Benton						
Field	104	22	−7.35	<0.001	2.10	<0.001
Laboratory	416	361	−3.93	<0.001	1.11	<0.001
Bear Ridge						
Field	32	9	−3.26	0.0087	1.16	0.0044
Laboratory	51	23	−4.55	<0.001	2.22	<0.001
Cold Springs						
Field	59	14	−6.97	<0.001	1.51	<0.001
Laboratory	125	56	−6.91	<0.001	1.55	<0.001

Note. N is the number of injections given to the group and used in the analysis; N_i is the number of individuals actually used; α and β are the intercept and slope of the curvilinear regression respectively given with their significance level (P).

RESULTS

Curvilinear regression for the field and laboratory groups from each population was significant at the $\alpha = 0.05$ level (Table 1). Bear Ridge had the lowest estimated 50% doses for both neonate and field-caught groups (6.78 and 6.03 MAMU, respectively), followed by Benton (33.65 and 31.84 MAMU), and then Cold Springs (86.47 and 99.27 MAMU). The same pattern was observed for δ, with the smallest absolute values calculated for Bear Ridge and the largest for Cold Springs (Table 2). The level of matching between field-caught and laboratory estimates can be visualized by constructing the paired dose-response curves with their respective 50% TTX doses for all three populations (Figure 1).

TABLE 2. ESTIMATES FOR GROUP 50% TTX DOSES AND THE DIFFERENCE BETWEEN THE ESTIMATES FOR THE LABORATORY (NEONATE) AND FIELD ANIMALS

	50% Dose (MAMU)		$\delta(P < 0.05)$	
Population	Field (τ_f)	Laboratory (τ_n)	Minimum	Maximum
Bear Ridge	6.03 (3.87, 9.17)	6.78 (5.58, 8.20)	0.65	0.84
Benton	31.84 (25.16, 40.23)	33.65 (30.42, 40.39)	1.62	2.00
Cold Springs	99.27 (67.97, 144.78)	86.47 (70.33, 106.26)	−11.84	−13.75

Note. Group 50% doses (τ_f and τ_n) are given with their 95% confidence interval in parentheses. The difference between groups (δ) is given as the minimum/maximum value for the difference based on bootstrapping analysis.

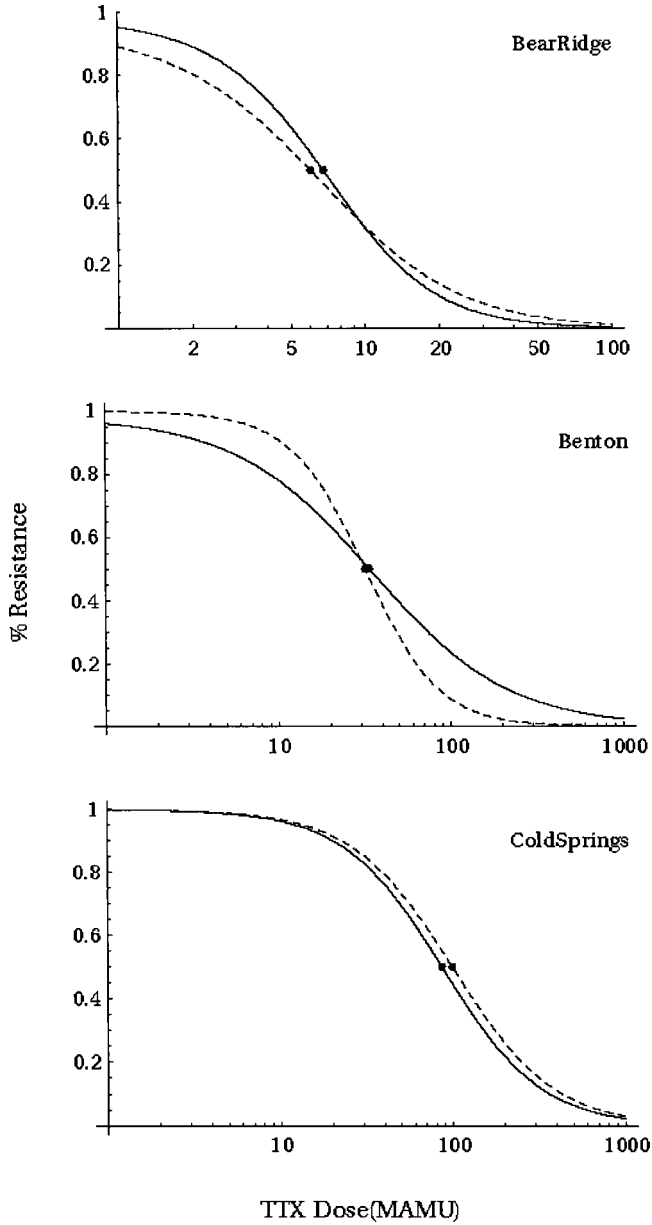

FIG. 1. Population dose-response curves for TTX resistance in neonate (—) and field-caught (- - -) garter snakes. The • indicates the estimated 50% dose for the group. The dose-response curves were estimated using curvilinear regression techniques (see text).

DISCUSSION

The use of field-caught snakes provides a comparable alternative to laboratory-reared neonates for bioassays of TTX resistance in the two species of *Thamnophis* tested. Field-caught and neonate estimates obtained from the populations from Bear Ridge, Benton (both *T. sirtalis*) and Cold Springs (*T. couchii*) produced nearly identical dose-response curves (Figure 1). More importantly for quantitative comparisons, the estimated 50% doses for both populations are similar (Table 2). For Benton, the estimated 50% doses are less than 6% different ($\delta \max/\tau_n$). For Bear Ridge and Cold Springs, the estimated 50% doses are approximately 13% different and 16% different, respectively. The increased precision of the Benton sample is probably due to the large sample size. These populations provide evidence that using field-caught garter snakes for TTX bioassays provides data similar to assays performed on laboratory-born neonate *Thamnophis*.

By comparing the values of δ, it appears there is no bias in the difference between the field and laboratory estimates. Benton and Bear Ridge both exhibit positive values of δ (though both are relatively small). Cold Springs on the other hand exhibited a negative deviation in field and laboratory estimates. The Cold Springs estimate does, however, exhibit the largest deviation of all three populations; this larger value is most likely due to the nature of the analysis (discussed later) rather than a systematic bias. More observations are needed to show conclusively that the estimation of a population 50% dose from field-caught individuals is unbiased.

Because the technique of estimating 50% doses utilizes a linear regression performed on appropriately transformed variables, sampling regime plays an important role in obtaining accurate results. In order to obtain good regression results, sufficient variation in the x-variable and appropriate sample sizes are needed. As an example of the importance of sampling regime, data that were collected from Benton field-caught snakes prior to the current study were analyzed (not shown). This group of snakes ($N = 31$) was given doses that ranged only between 23 and 31 MAMUs (as compared to the 5–100 MAMUs given to the Benton field-caught snakes for this study). Because of the lack of variation in dose, a non-significant regression result was obtained ($P_\alpha = 0.1962$, $P_\beta = 0.2060$). The 50% dose estimated for this group was 26.85 MAMU (22.23, 32.40). This estimate, therefore, had δ values that were more than three times as large as those estimated for the current study ($6.69 < \delta < 6.91$). Without a proper sampling scheme to produce a significant curvilinear regression, it is impossible to estimate accurately the population dose-response curve (Figure 2). Future assays for TTX resistance in garter snakes should employ a sampling regime over a broad range of doses to provide sufficient variation in the x-variable. Ideally the distribution of doses should be uniform about the 50% dose, and the tails of the distribution should encompass doses that produce resistance measures greater than 80% and doses that produce

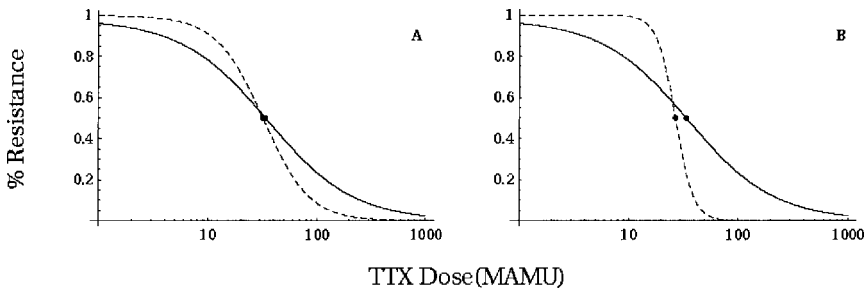

FIG. 2. A graphical comparison of field-caught animals from Benton, OR. **A** and **B** show the estimated 50% dose and dose-response curves for the field-caught animals (- - -) used for this study and a prior sample respectively. Estimates using the data collected for this study (**A**) were done using a proper sampling regime and more closely match the neonate estimates (——). The prior sample (**B**) was tested over a restricted range of doses near the known 50% dose.

resistance measures of less than 20% (higher doses may damage animals). Multiple doses either above the 80% level or below the 20% level should be avoided, as they reduce the accuracy and provide no new information. If such "repetitive" doses exist, it is suggested that they be dropped from the analysis.

Sample size also plays a critical role in achieving good results using this technique. The number of injections used in the curvilinear regression is inflated over the number of actual individuals used for the study (Table 1). However, the use of field-caught animals alleviates some of the sample-size issues that occur when using neonates. Field-caught animals, assuming littermates are not caught, provide genetically independent data points, while multiple littermates are typically used in neonate analyses. For example, 125 total injections were given to 56 neonates for the estimation of the Cold Springs data, but the 56 neonates came from 5 litters. Brodie and Brodie (1991) have demonstrated a genetic basis to TTX resistance in *T. sirtalis*. The neonate estimate for Cold Springs may, thus, represent a smaller, more biased, sample than the 14 field-caught individuals used for the analysis. The 50% dose estimate for field-caught snakes from Cold Springs may actually lie closer to the population mean, though fewer injections (59) were used in the analysis.

The importance of large sample size increases in tandem with resistance levels. The spread of the estimated difference exhibits a heteroscedastic pattern, with small variance at low 50% doses and large variance at large 50% doses. This pattern results from using natural log transformed doses prior to regression. The estimates for Cold Springs, Benton, and Bear Ridge are exemplary of this behavior. The regression estimates for the 50% dose for the field and laboratory groups from all three populations showed almost identical levels of difference after

analysis (approximately 0.05–0.14). However, upon reversing the transform of x (MAMU) to $\ln(x)$ (ln MAMU), the values of δ for Cold Springs are approximately six times as large as those for Benton, and almost 12 times as large as those for Bear Ridge (Table 2); though δ for Benton is larger than δ for Bear Ridge, Benton estimates actually showed the least deviation prior to the reverse transform. This discrepancy is because the 50% dose of Cold Springs (86.47 MAMU) is larger than that of Benton (33.65 MAMU) and much larger than that of Bear Ridge (6.78 MAMU). This behavior implies that larger sample sizes and better sampling regimes are critical to produce accurate estimates for populations that are more resistant to TTX.

The use of multiple injections on one individual for the analysis introduces the issue of pseudoreplication. Because the injections outnumbered the individuals, the degrees of freedom for the analysis are exaggerated in a standard regression. The same type of analysis can be performed using a mixed linear model, where individuals represent a random effect (i.e., individuals have different dose-response curves but are chosen at random from the population). The effect of using a mixed linear model is a reduction in the degrees of freedom associated with the estimation of the parameters, but the variance due to individual differences is removed producing better (i.e., lower variance) parameters. For populations where this type of analysis has been performed, the effect on the estimation of the 50% dose is negligible. For example, for the field-caught Bear Ridge animals, the degrees of freedom dropped from 31 to 22, the 50% dose only changed from 6.03–6.07 MAMU, and the variance of the 50% dose estimate decreased. The use of a mixed linear model is advantageous for proper parameter estimation but makes little difference in the estimation of the 50% dose. We have chosen to use linear regression in this paper because prior work used linear regression techniques (cf. Brodie et al., 2002).

The deviations observed between field-caught and neonate estimates could be due to natural selection but are more likely the result of statistical inaccuracy. The broad-sense heritability, an upper-bound to narrow-sense heritability, of TTX resistance for a population of *T. sirtalis* near Benton was estimated to be 0.715 ± 0.162 (Brodie and Brodie, 1990). In combination, the observed deviations and the potentially high heritability of resistance would imply rapidly shifting TTX resistance levels in some populations. The larger negative deviation observed at Cold Springs could be due to a cost of resistance (Brodie and Brodie, 1999), but such a drastic drop in resistance levels from birth to adulthood makes this explanation unlikely. Ideally, the method of using field-caught garter snakes could lead to producing measures of selection through "cross-sectional" studies (cf. Lande and Arnold, 1983). Being able to perform such a study would be of great benefit because the use of catch-and-release studies for studying selection in *Thamnophis* is problematic.

We achieved equivalent estimates of TTX resistance in both field-caught and laboratory animals by using a mass-adjusted dose. Because of the correlation

between mass and resistance, we gain insight into developmental changes in resistance as snakes mature by looking at the change in mass through ontogeny. This pattern may be further extended to muscular development because the mechanism for TTX resistance is thought to be altered sodium channel morphology in muscle tissue (Geffeney et al., 2002). Selection for TTX resistance *within* a population may favor either faster size development or larger overall size, depending on the timing of selection and assuming that the mass-resistance correlation is not purely phenotypic. The positive covariance of mass and resistance has not been examined across populations.

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FIRST IDENTIFICATION OF A PUTATIVE SEX PHEROMONE IN A PRAYING MANTID

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Abstract—Praying mantids are models for a wide variety of behavioral, physiological, and ecological studies, and sex pheromones have been assumed to be important components of their biology. However, no mantid pheromone has ever been identified. We collected volatiles emitted by females of the mantid, *Sphodromantis lineola*, via solid phase microextraction (SPME). Mass spectral analysis revealed the collected volatiles to be a mixture of pentadecanal and tetradecanal. We prepared a synthetic mixture of these compounds, and found that males were both attracted to this mixture and stimulated to exhibit typical precopulatory behavior. We then examined male antennae with scanning electron microscopy, and confirmed the presence of porous antennal sensilla typical of insect pheromone receptors, i.e., that male mantids are equipped with the appropriate morphological apparatus to receive volatile chemical signals. Pheromones, in conjunction with visual and tactile cues, are thus an important feature of the reproductive biology of this, and undoubtedly other species of mantids. In addition to adding a crucial aspect of behavioral biology to our knowledge of this group, identification and synthesis of mantid pheromones may be a first step in attracting and aggregating these generalist predators for use in pest control.

Key Words—behavioral assay, insect mating behavior, sex attractants, *Sphodromantis lineola*.

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INTRODUCTION

Sex pheromones are far better documented for herbivorous insects than for predators, mainly because of herbivores' economic importance as pests (Landolt, 1997). However, predaceous insects can be important pest control agents (DeBach, 1974), and praying mantids (order Dictyoptera, suborder Mantodea) may prove to be effective in this regard. Natural densities of mantids can exert strong influences on arthropod assemblages and, through trophic cascades, on plant productivity as well (Fagan and Hurd 1994; Moran et al., 1996; Moran and Hurd, 1998). An important precursor to the effective use of mantids and other predators in biological control is discovering how to attract and aggregate them to increase their effective density, for example, by using pheromones. Praying mantids comprise more than 1500 species worldwide (Arnett, 1993) and have a rich descriptive literature devoted to their reproductive behavior (Prete and Wolfe, 1992; Prete, 1995). However, references to mantid pheromones are entirely circumstantial, based on inferences from observations of behavior or morphology (Robinson and Robinson, 1979; Maxwell, 1999). We addressed three key questions: (i) Do females produce an identifiable, pheromone-like chemical that can be synthesized; (ii) If so, does this chemical have a measurable effect on male behavior; (iii) Do males possess antennal sensilla (Zacharuk, 1985) morphologically identifiable as chemoreceptors for pheromones?

METHODS AND MATERIALS

Insects. The mantid, *Sphodromantis lineola* (Burmeister), is widely distributed in western Africa (Edmunds, 1972; Kumar, 1973). Among mantids, *S. lineola* is relatively large, with minor sexual dimorphism. Individuals were hatched and raised in the laboratory according to the methods detailed in Prete (1999). Adults were housed and fed separately, assuring that both males and females were sexually naive.

Collection of Volatiles. We collected volatile chemicals from the headspace of individual adult female mantids using a solid phase microextraction (SPME) apparatus (Supelco Inc. Deerfield, IL) with a polydimethylsiloxane coated (100 μm) fiber (Malosse et al., 1995). The SPME holder was inserted into a beaker containing a virgin female mantid, where the fiber was exposed for 10–12 hr under low ambient lighting. This procedure was repeated for three separate females that were demonstrably attractive to males in the laboratory, i.e., males placed in proximity had attempted to copulate with them but were prevented from doing so. Other than that they attract males, there is no known overt behavior associated with pheromone emission by females of this species (e.g., abdominal flexion or other posturing), so we collected emitted volatiles for nearly the entire day to

maximize our probability of success. Females' activity period is diurnal, so we did not attempt to collect pheromones during scotophase. Mantids are by nature sedentary insects, and females quickly became accustomed to their confinement, such that normal preening behavior could be observed.

Mass spectra were obtained with a Shimadzu QP-5000 GC/MS in the EI mode, equipped with a Rtx-5, 30 m × 0.32 mm column (SGE, Austrilia). The SPME fiber was thermally desorbed at 260°C for 1.5 min in the splitless mode. The oven was then held at 60°C for 3 min, programmed at 10°C per min to 250°C, and held for 30 min.

Following identification of the constituents collected by SPME, synthetic chemicals were tested in the behavioral assay. Tetradecanal was obtained from Fluka Chemical Co. and pentadecanal was obtained by pyridinium chlorochromate oxidation of pentadecanol followed by vacuum distillation, and was >98% pure by GC/MS analysis.

Biological Assay. Bioassays of the putative pheromone mixture were carried out when we knew the mantids were sexually active, as evidenced by individuals from the same cohort attracting each other and copulating. We tested the effect of our reconstructed female-produced pheromone on males in two behavioral bioassays, a modified Y-maze (Figure 1), and an open-field behavioral assay. We predicted that the pheromone would attract males into the Y-maze corridor containing the pheromone (versus the control corridor), and that it would cause males to perform precopulatory (so-called "courting") behavior in the absence of females.

In the Y-maze experiment, eight male *S. lineola* were placed individually in an uncovered 10 × 11.5 cm plastic jar, the mouth of which was inserted into an opening at one end of a 46-cm long × 40-cm wide × 11.5-cm high, white, glass-covered arena with styrofoam walls and a cardboard floor (Figure 1). A V-shaped interior partition with its apex pointed toward the mantid divided the rear three quarters of the arena space into two 34-cm corridors. An aquarium pump created air flow of ca. 2500 cc per min emanating from a hole in the center of the far wall of each corridor. The room was illuminated by overhead fluorescent bulbs and ambient light from a north window.

At the beginning of each test day, each of two 1.25 × 2.5 cm strips of filter paper were soaked in either a solvent (anhydrous chloroform) or the solvent plus the synthesized pheromone: 40 mg of aldehydes in a 1:3 ratio (see above) per 5 ml solvent, and air dried. Each was then placed in a watch glass sitting under the air hole at the end of each corridor. Mantids were videotaped during the tests.

We ran eight 2-hr trials over a 5-d period (1–3 trials per d), and calculated the amount of time each mantid spent in each of the corridors and in both the front halves (Figure 1, sectors V1 and Ph1) and rear halves of the corridors (sectors V2 and Ph2). We expressed the time spent in a corridor or sector as the proportion of

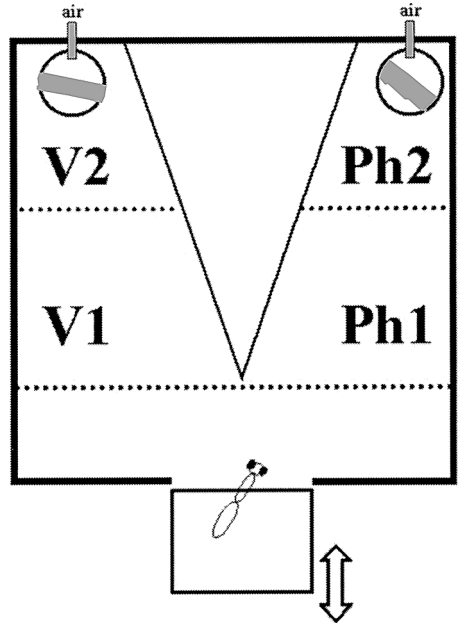

FIG. 1. Schematic of the Y-maze used to test the effectiveness of the synthesized female pheromone. An aquarium pump created an air flow over watch glasses holding filter paper strips treated with a solvent (V) or the putative pheromone (Ph, a 1:3 ratio of tetradecanal and pentadecanal) dissolved in the solvent and allowed to dry. Corridors were divided into front halves (sectors V1 and Ph1) and rear halves (sectors V2 and Ph2).

the total time spent in the pair of corresponding areas, and considered the mantid as having chosen an area when this value exceeded 75%.

In the open-field assay, three males were placed in the center of a 40- cm^2 glass-covered white arena with Styrofoam walls and a cardboard floor. A dummy mantid the size of a female, made out of a translucent white plastic tube with wooden toothpick legs, stood in each corner. Two dummies had a solvent-treated (1×4 cm) strip of filter paper taped to their dorsal sides, whereas the other two had strips of filter paper treated with the solvent plus the putative pheromone. The males were videotaped for 6 hr under dim illumination, and the video was analyzed for three behaviors: (i) aggregation on or around one of the dummies; (ii) "flying leaps," the characteristic, precopulatory mounting attempts made by males (Kynaston et al., 1994); (iii) abdominal S-bending, the distinctive sideways abdominal flexing associated with precopulatory behavior (Liske, 1991). We considered only vigorous, unequivocal S-bends in which the abdomen flexed more than 15° from the midline and which were accompanied by the characteristic, precopulatory "searching movements" of the abdomen tip (Liske, 1999; Maxwell, 1999).

Antennal Morphology. We examined the antennae of eight males with both light (16 antennae) and electron microscopy (7 antennae). In the former case, we used ethanol preserved specimens. In the latter case, two protocols were used. In the first, mantids were immobilized with acetone vapor after which the heads were fixed in 3% glutaraldehyde in 0.05 M cacodylate buffer, washed in buffer, and dehydrated in a graded series of ethanol baths. Heads were then submerged in hexamethyldisilazane for 10 min and allowed to air dry overnight in a desiccator. Antennae were cut into thirds, mounted, sputter coated with AuPd, and viewed with a JOEL T200 scanning electron microscope (SEM). In the second protocol, mantids were euthanized and fixed with 70% ethanol. Antennae were removed, mounted on a 25-mm aluminum stub using either adhesive carbon tape or carbon adhesive tabs, sputter coated with gold, and viewed with an Hitachi S-2300 SEM. Prior to mounting, some antennae were pretreated to remove surface dirt and wax.

To estimate the number of sensilla, we counted those in a narrow median strip on each of 12 antennal segments, or flagellomeres (the terminal flagellomere [tip] and every 10th flagellomere from #10–#110) of a single antenna using SEM micrographs (300×). We then multiplied that number by the ratio of the total surface area of each flagellomere to the area of the median strip. For flagellomeres #10–110, the total number per flagellomere was multiplied to account for intermediate flagellomeres, and that total was added to the estimated number for the tip. This procedure was modified from Slifer (1968) and resulted in comparable numbers to those she estimated for male *Tenodera sinensis* (i.e., 38,250), the only other species for which there are data.

RESULTS

Pheromone Identification. The SPME GC/MS analysis of the three female mantids used in our study gave identical results, showing eight consistent volatile components (Figure 2). The mass spectra of peaks 3 and 5 suggested that they were tetradecanal and pentadecanal, respectively (NIST/EPA/NIH, 1999). A single ion search for the ion at $m/z = 82$, common to straight chain aldehydes, showed a small peak at 14.5 min that corresponded to the retention time of tridecanal. The mass spectra of the other small peaks showing $m/z = 82$ were not consistent with straight chain aldehydes. Peak 4 is heptadecane, a common metabolite of stearic acid in insects and unlikely to be used as a pheromone. The remaining peaks were artefacts: peak 2 was a phthallate plasticizer, and the rest of the peaks were products of degradation of the SPME coating. The GC/MS traces suggested a 1:3 ratio of tetradecanal:pentadecanal, and GC/MS analysis of an SPME sample of both aldehyde standards confirmed their identification.

Behavioral Assay. The results of both behavioral tests were unequivocal. In the first test, five of the eight male mantids made a single excursion, and one made

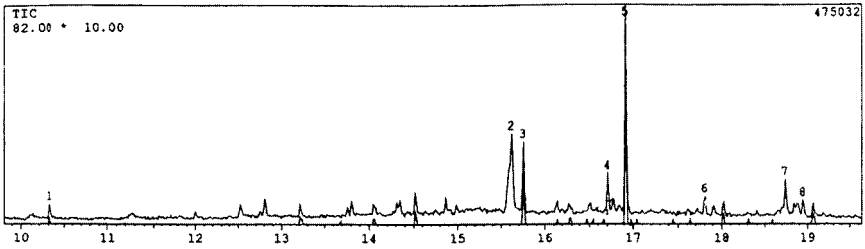

FIG. 2. Total ion chromatograms of SPME sample from a female *S. lineola* (toptrace) and synthetic standards (bottom trace). Units on *x*-axis are minutes. Peak number 3 is tetradecanal; peak 5 is pentadecanal. Other numbered peaks are explained in text.

two excursions out of the jar directly into the pheromone corridor (Figure 1, Ph) and then returned to the mouth of the jar where they remained perched for the remainder of the trial. The seventh made a single excursion out of the jar which included a brief (42 sec) visit to the proximal end of the solvent corridor (V1) after which it moved into the pheromone corridor (Ph) before returning to the jar. The eighth male made several excursions into the corridors but only one included a brief (4.3 min) visit to the solvent corridor (V). The six mantids that visited only the pheromone corridor entered the front half (Ph1) within an average time of 10.1 $\pm$ 8.6 min (mean $\pm$ SD) after the beginning of the trial. The two males that briefly visited the solvent corridor moved from its front half (V1) into Ph1 in just 16 and 48 sec, respectively. By our criterion, all males chose the pheromone corridor. The binomial probability of eight such choices by chance is 0.0039. The insects spent an average of 98.6% ($\pm$3.5 SD) of their corridor time in the pheromone corridor, 97.5% ($\pm$4.8 SD) of their time in the front halves of the corridors within Ph1, and 99.7% ($\pm$0.8 SD) of their time in the rear halves of the corridors within Ph2 (corresponding mean times = 37.9, 22.2, and 15.7 min, respectively).

As we originally hypothesized, males displayed vigorous precopulatory behavior in the open-field assay. However, there is a substantial amount of anecdotal data (e.g., Liske, 1991; Maxwell, 1999) indicating that olfactory cues work synergistically with visual cues provided by a live female mantid during the pre- and copulatory phases of mating behavior. Although the chemical cue was sufficient to elicit vigorous precopulatory behavior, dummy mantids lacked visual cues that could have been provided by a live female and which might have elicited the complete sequence of mating behaviours. However, the presence of live males in association with the chemical cue caused males to direct their precopulatory behaviors toward each other. During the bioassays males displayed no aggregation behavior, but the group exposed to the putative pheromone preparation all repeatedly performed precopulatory behaviors during the first 230 min of the 360 min test. This behavior was not seen in males exposed to only the solvent. Each of

the three mantids performed at least one flying leap, and each of the five leaps that occurred was a clear attempt to mount another male. None of the leaps was associated with aggressive behaviors, e.g., deimatic displays, chasing, or defensive striking (Maldonado,1970; Prete and Wolfe, 1992; Prete et al., 1999). In three cases, the leaps were combined with other mating related behaviors. At 51 min mantid #3 leaped onto another male and then aligned himself parallel to his partner as he would on a female. At 129 and 202 min, mantid #3 leaped onto another male after performing a series of vigorous S-bends and a slow stalking approach, as is done in normal male–female precopulatory encounters. After each of the five leaps, the bottom male walked out from under the leaper, or the leaper dismounted and fluttered to the arena floor as they do when dismounting a female. Failures to remain mounted may reflect the absence of female-related tactile and/or contact chemosensory feedback.

The most dramatic display of precopulatory behavior was the vigorous S-bending performed by each of the males. In most cases, the abdominal bends approached 90° and were accompanied by "searching" movements of the abdomen tip (Liske, 1991). In all but one case (at 229 min), the performing male was facing at least one other male. S-bending behavior occurred in bouts which could include as many as six bends in rapid succession and each S-bend usually included several "reaches" (smaller decreases and increases in the degree of flexion) with searching movements superimposed on the reaches. In addition, most of the S-bends were accompanied by side-stepping movements in the direction of the bend as if the male were creeping toward and reaching for the target male. Interestingly, one S-bending bout (at129 min) was followed by a flying leap onto another male, and a second bout (at 135 min) occurred after a male had mounted one of the pheromone dummies.

Antennal Morphology. The antennae of *S. lineola* consist of two basal segments (pedicel and scape) and a tapering, filiform segment, the flagellum, which, in males, is divided into an average of 118 (±3.16 SD) segment-like flagellomeres. Following convention, we numbered the flagellomeres beginning at the tip (Chapman and Greenwood, 1986). In our sample, mean antenna length was 31(±1.55 SD) mm, or about 52% of body length (head to tip of abdomen). Basal flagellomeres were wider (215 μm) than long (133 μm). More distally, flagellomeres became progressively longer, reaching a maximum of 337 μm at antennal midpoint, and progressively thinner toward the tip (50 μm wide by 227 μm long).

We estimated that a single antenna held a total of 29,828 hair- and peg-like sensilla that could be divided into three distinct, well-recognized morphological types, each of which contained subtypes that appear analogous to chemoreceptors in other insects (Zacharuk, 1985). The three morphological classes were (i) large trichoid sensilla, (ii) small trichoid sensilla, and (iii) grooved basiconic sensilla. The large trichoid sensilla are apparently identical to Slifer's (1968) two classes of "thick-walled chemoreceptors." The small trichoid sensilla correspond to the two

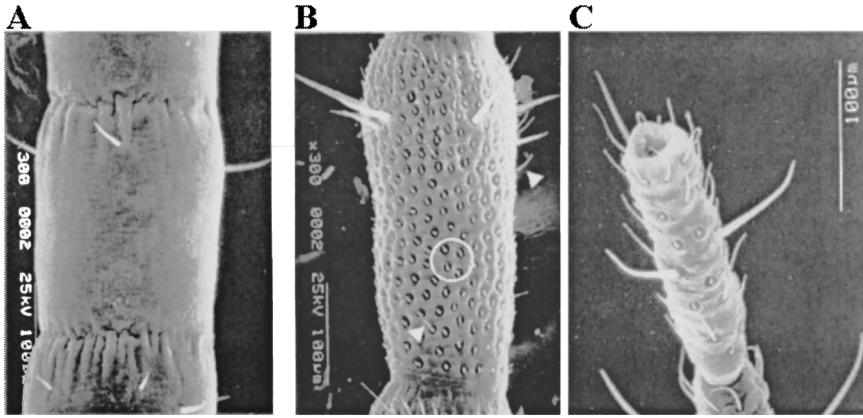

FIG. 3. SEM micrographs (×300) of flagellomeres at three locations on the antenna of male *S. lineola*. (a) Flagellomere #100 (tip = #1) with a ring of large trichoid sensilla around the distal end; (b) Flagellomere #60 (mid antenna). Large trichoids encircle the distal end and small trichoids (arrowheads) are concentrated within the distal and proximal thirds of the flagellomere. Basiconic sensilla (encircled) blanket the flagellomere at densities as high as 3420 per mm^2; (c) Antennal tip showing all three sensilla types.

classes of small trichoid sensilla that Slifer called "medium thin-walled" and "long thin-walled chemoreceptors." On the basis of her descriptions, these correspond to what are now called thin- and thick-walled, respectively, multiporous receptors with pitted surfaces (Zacharuk, 1985). These small trichoid sensilla matched Slifer's in distribution pattern and overall number but were generally longer than those she found on *Tenodera* (i.e., 24–46 μm versus 14–45 μm).

The large, tapered trichoid sensilla were set in a circle around the distal third of each flagellomere (4–7 per flagellomere) beginning at approximately the 110th flagellomere. We estimated a total of 597 per antenna (Figure 3a). All measured 9 μm diam at their base but were shortest (33–35 μm) on the basal most flagellomeres and longest (119–128 μm) at the antennal tip (Figure 3b and c). The large trichoid sensilla were corrugated by longitudinal ridges and one third of those examined had an unelaborated 0.5–.75 μm pore at their apices (Figure 4a). Trichoid sensilla with apical pores are generally contact chemoreceptors (Zacharuk, 1985). In mantids, these may be involved in chemoreception during male antennal drumming of females prior to and during copulation. Also, the large trichoid sensillae appeared to be inserted in a cuticular protuberance, suggesting they may also have a mechanosensory function.

Beginning at approximately the 90th flagellomere, small trichoid sensilla (3 μm diam at the base by 24–46 μm long) appeared around the basal and distal thirds of each flagellomere. We estimated 3118 per antenna (Figure 3b).

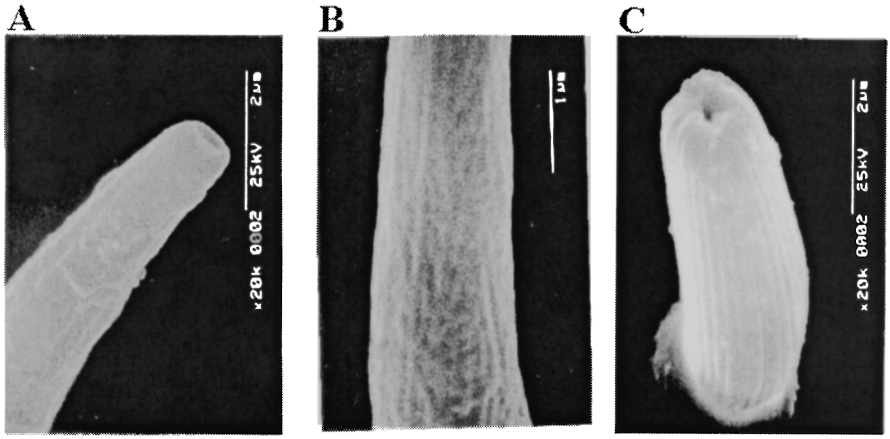

FIG. 4. SEM micrographs ($\times$ 20k) of three antennal sensilla subtypes containing pores. (a) The tip of a large trichoid sensilla with an apical pore; (b) The pitted surface of a small trichoid sensilla; (c) A grooved basiconic sensilla with an apical pore.

The surfaces of all the small trichoids that we examined were covered with pits (Figure 4b) suggesting that they are typical insect multiporous olfactory sensilla (Dickens et al., 1998). Also, beginning at approximately the 90th flagellomere, we found a rich array of small (1.5 μm basal diam by 6 μm long), longitudinally grooved basiconic sensilla that covered the flagellomeres at densities as high as 3420 per mm^2 (Figure 3b and c). We estimated a total of 26,113 of these sensilla per antenna. The tips of the basiconic sensilla were either blunt and rounded or had an apical slit or pore (Figure 4c). Although grooved basiconic sensilla in other insects have rows of pores in their longitudinal grooves, side pores were not evident in any of our preparations even at 80K magnification. Grooved basiconic sensilla are characteristic olfactory receptors found on the antennae of a variety of insects and have a number of olfactory functions and specificities, including pheromone perception (Chapman and Greenwood, 1986). Although the sensilla functions must be confirmed electrophysiologically, their distinguishing morphological characteristics strongly suggest that male *S. lineola* have the capacity for pheromone perception via characteristic antennal sensilla.

DISCUSSION

The simple aldehyde components of the pheromone we collected were unexpected because tetradecanal and pentadecanal have been reported only as minor components of putative pheromone blends of other insects (Kalo, 1979; Klun et al., 1980; Farine et al., 1992; McDaniel at al., 1992; Bartelt et al., 1993; Sant'ana et al.,

1999). Because SPME fibers are not uniformly sensitive to all compounds, the ratio of the two aldehydes used in our behavioral assays (based on the sizes of the GC peaks) may not precisely match the ratio produced by female *S. lineola* (Bartelt, 1997), which also may include a trace of tridecanal. However, our behavioral assays indicated that the reconstructed mixture may approximate the actual pheromone. Male mantids are initially attracted to females by female-produced pheromones, and premating behaviors include vigorous antennal movements directed toward females and antennal drumming of the female cuticle (e.g., Kynaston et al., 1994; Maxwell, 1999). Data presented here support this hypothesis and the generally held hypothesis that although pheromones act as attractants, they do not play an exclusive role in mantid mating behavior. Apparently, olfactory signals are integrated with visual and tactile cues during the mating process. Thus, lone males attracted into the pheromone corridor in the Y-maze experiment did not display precopulatory behavior following attraction, and males caged together without females (and, hence, without female-related cues) directed their precopulatory behaviors toward each other. The importance of visual and tactile cues in mantid mating behavior is also evidenced, respectively, by the facts that anosmic males (i.e., males with no antennae) will mate if placed near a female, and decapitated males can mate once they are mounted (e.g., Rau and Rau,1913; Roeder, 1963). The precise nature of these non-olfactory signals and how they interact with volatile sex attractants has yet to be elucidated. However, tactile signals may include contact chemoreception of the long-chain aldehyde constituents found in mantid cuticles (Jones et al., 1997).

Pheromones may play a broader role in mantid behavior than sex recognition alone. Female mantids often aggregate during the egg-laying portion of their life cycle, possibly by responding to each others' pheromones (Hurd,1999). This could be selectively advantageous in that the augmented concentration of pheromone produced by multiple females would be more attractive to males (a single male can mate with multiple females), and the supply of males often diminishes toward the end of the life cycle (Hurd et al., 1994). This is especially plausible in that pheromones of other orthopteroid insects have been shown to attract both sexes (Jacobson, 1972). The potential for a single stimulus to elicit different behaviors in the two sexes also is consistent with the findings of Prete et al. (2002) that male and female *S. lineola* responded to the same visual stimuli in different ways. If both sexes are attracted (even if for different reasons), then using the synthesized pheromone to attract mantids for biological control purposes might be doubly effective, because females have larger appetites than males (Hurd, 1999) and the total density of males and females would be greater than that of either sex alone.

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EVIDENCE FOR A MALE-PRODUCED SEX PHEROMONE IN THE WESTERN FLOWER THRIPS *Frankliniella occidentalis*

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Abstract—Olfactometer bioassays of walking adult western flower thrips *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) showed that virgin females (1- to 3-d postemergence) were attracted to the odor of 25 adult males, but not to the odor of 25 adult females, providing behavioral evidence for a male-produced sex pheromone in this species. In contrast to earlier findings, mixed-age adult males were attracted to the odor of adult males. GC analysis of odors collected on SPME fibers revealed two major components and five minor components that were present in the male odor and not in the female odor. The compounds were not present in hexane extracts of males, indicating that these compounds are produced on demand and not stored.

Key Words—*Frankliniella occidentalis*, Thysanoptera, Thripidae, sex pheromone, mating, aggregation, sternal gland, olfactometer, integrated pest management.

INTRODUCTION

In many species of thrips, including the western flower thrips *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae), adult males possess a series of abdominal "sternal glands," which are structurally consistent with pheromone release (Bode, 1978; Moritz, 1997). Circumstantial evidence suggests that the glands produce a sex pheromone that calms females at very close range (Pelikan, 1951). However, the discovery that adult female *Frankliniella schultzei* respond when walking or in flight to the odor of adult males provides evidence for an attractant role (Milne, 1997; Milne et al., 2002). de Kogel and van Deventer (2003) have since provided behavioral evidence for a male-produced sex pheromone in *F. occidentalis* that attracts females but not males. This species forms male

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aggregations, within which there are aggressive male–male interactions and females arrive, mate, and leave immediately (Terry and Gardner, 1990; Terry and Dyreson, 1996; Terry, 1997). A male-produced sex pheromone could have many roles in the complex interactions within and between the sexes at aggregation sites.

No information on the chemical identity of a sex pheromone has yet been published for any species of thrips (Terry, 1997), although anal secretions have been identified in many species (Suzuki et al., 2000). In *F. occidentalis*, the larvae, but not adults, produce an anal droplet consisting of decyl acetate and dodecyl acetate, which acts as an alarm pheromone and has many effects on the behavior of both larvae and adults (Teerling et al., 1993; MacDonald et al., 2002, 2003).

This paper confirms and extends the behavioral evidence for a male-produced sex attractant pheromone in *F. occidentalis* and provides chemical evidence of male-produced volatiles that are possible pheromone components. A synthetic sex pheromone could improve the monitoring or integrated pest management of this species, which has spread since the 1970s to become a major worldwide pest of horticulture and agriculture (Kirk, 2002; Kirk and Terry, in press).

METHODS AND MATERIALS

Thrips Rearing. A culture of *F. occidentalis* from commercial glasshouses in the United Kingdom was maintained on pot chrysanthemums (*Dendranthema grandiflora* Tzvelev) at $25 \pm 2^{\circ}$C and L18:D6. Adult thrips collected arbitrarily from this culture are described as "mixed age." To obtain known-age virgin females, adults were transferred from the chrysanthemums to 500-ml plastic pots (Nalgene, Nalge Ltd., UK) containing French bean pods (*Phaseolus vulgaris* L.) and pine pollen (*Pinus brutia* Tenore) and kept at $25 \pm 0.3^{\circ}$C and L18:D6. A hole (diam. 70 mm) in the lid allowed moisture exchange through a piece of tissue (Kimwipes Lite, Kimberley-Clark, U.K.) held in place between the lid and the pot. The resulting second-instars were isolated, to prevent later mating, by moving them to individual Eppendorf tubes containing a section of bean pod sealed with paraffin wax at each end. A hole in the lid (diam. 5 mm) allowed moisture exchange through a piece of tissue between the lid and the tube. Daily observations of the tubes allowed the day of adult emergence to be recorded.

Y-Tube Olfactometer. A glass Y-tube with branches at an angle of 90° and stem and branches of length 60 mm and internal diam 5 mm was held horizontally. Zero-grade air from a cylinder (British Oxygen Corporation, Manchester) was passed through an activated charcoal filter, separated into two streams, and then flow-rate regulated by two rotameters (Supelco, U.K.) to a speed of 50 mm sec^{-1} in each branch of the Y-tube and 100 mm sec^{-1} in the stem. A 50-ml round glass flask with a Drechsel head before each branch of the Y-tube provided test and control odor sources. Small pieces of glass wool in the inlet and outlet tubes prevented

thrips leaving the flask. Each flask had a semicircle of filter paper (Whatman No. 1, diam. 42.5 mm) kept moistened with distilled water inside it, and was illuminated from above by four fluorescent tubes and one arm of a fiber-optic cold light source at a distance of 40 mm (about 10,000 lux). These conditions were used because they produced the patrolling and fighting behavior shown by males at aggregations (Terry and Gardner, 1990), which was considered most likely to induce pheromone production. Thrips remained active for at least 6 hr. Connections were made with Teflon tubing and brass Swagelok® connectors. The Y-tube was screened on all sides from visual influences by a 100-mm high wall of matt black card and illuminated from above by four fluorescent tubes (about 1900 lux). A similar Y-tube olfactometer system worked well for assessing the responses of *F. occidentalis* to volatile plant compounds (Koschier et al., 2000).

Bioassays. Experiments were carried out at 25 ± 1°C. Odor-source thrips (25 mixed-age adults) were collected with a small aspirator, anesthetized briefly with carbon dioxide, checked for gender under a dissecting microscope, then counted into the treatment flask. Test thrips were transferred individually from plants to the base of the Y-tube with an aspirator and a fine brush and were not anesthetized. They walked up the tube and a "choice" was recorded when they crossed a line 20 mm down either branch. "No choice" was recorded if the line had not been crossed after 3 min for females or, because they took longer to run, 4 min for males. After a "choice" or a "no choice," each thrips was removed from the tube and placed at the base of the Y-tube again to be allowed to make a second choice. After every five or six thrips, the flasks, tubing, and Y-tube were turned 180° to reverse sides and minimize any side bias. About 20–30 individuals were tested in each run of an experiment. The apparatus was cleaned thoroughly before each run by rinsing with hexane. The glassware was also baked overnight at 200°C. Each experiment consisted of two or three runs.

Data Analysis. The bioassay data were analyzed with SPSS 11 (SPSS Inc., Chicago, Illinois, USA). "No choices" were excluded from the analysis. Data for first and second choices and for runs within an experiment were homogeneous ($P > 0.05$) in all cases and were combined. This was tested by a chi-square test with exact P values valid for small sample sizes. Responses were tested by a binomial test with exact two-tailed P values, with the null hypothesis that the two arms were chosen with equal probability.

Collection and Analysis of Volatiles. Mixed-age adult males or females were collected as described above for odor-source thrips and moved to a small glass flask sealed with a piece of Teflon tape. They were kept at 25–30°C and illuminated similarly to those in the bioassay so as to induce patrolling and fighting behavior. Headspace volatiles were collected on a 20-mm long 50/30 μm DVB/Carboxen SPME fiber assembly (57348-U, Supelco, U.K.). Collections were made for 4–5 hr from approximately 30–60 male or female thrips and repeated 10 times for males

and 5 times for females. Analyses of headspace volatiles collected from male or female thrips were preceded by analysis of headspace volatiles from the apparatus without the presence of thrips (blank control). SPME fibers were preconditioned by heating to 180°C for 2 hr before use. In addition, hexane extracts from five to hundreds of males were obtained by collecting males directly from chrysanthemum plants. Males were immersed in hexane and stored at −20°C until analysis.

Anal droplets were collected directly on SPME fibers from five second-instars to provide comparative retention time data for decyl acetate and dodecyl acetate, the components of the larval alarm pheromone (Teerling et al., 1993).

SPME samples and hexane extracts were analyzed on an Hewlett-Packard 5890 II+ gas chromatograph with an HP-5 capillary column, 30 m × 0.25 mm i.d., 0.25-mm film thickness. The sample was introduced via a septum-less heated injector (180°C) and the gas chromatograph (GC) was temperature programmed with an initial 2 min at 40°C, then an increase of 10°C min^{-1} to a final isothermal period at 250°C (10 min).

RESULTS

Bioassays. Virgin females (1- to 3-d postemergence) were attracted to the odor of 25 adult males, but not to the odor of 25 adult females (Table 1). Mixed-age adult females were also attracted to adult males and their preference for the odor side (66%) was similar to that of virgin females (70%). However, the response was not exclusively by females to males, because mixed-age males were also attracted to adult male odors. The preference of mixed-age adult males for males (66%) was similar to that of females for males.

Each thrips was tested twice in succession. For the odor sources that were preferred, there was no significant difference in preference between the first and

TABLE 1. RESPONSES OF WALKING ADULT *Frankliniella occidentalis* TO OTHER ADULTS OF THE SAME SPECIES IN A Y-TUBE OLFACTOMETER BIOASSAY[a]

Test insects[b]	Odor source[c]	Number of choices		Preference for odor side%	Probability
		Odor side	Control side		
Virgin females	Males	69	29	70	<0.001
Virgin females	Females	44	62	42	0.10
Females	Males	60	31	66	0.003
Males	Males	65	34	66	0.002

[a] Significance levels based on null hypothesis of equal preference for the two sides.
[b] All test insects were adults. Virgin females were 1- to 3-d postemergence and others were of mixed age.
[c] All odor sources consisted of 25 live adults of mixed age.

second choices ($P > 0.05$ for each experiment and each run of each experiment), indicating that responses occurred quickly, within minutes of encountering the odor, and were not improved by several minutes of additional prior exposure. There was also no significant correlation between the first and second choices of individual thrips for virgin females tested with odor from males ($P = 0.72$), mixed-age females tested with odor from males ($P = 0.49$) or males tested with odor from males ($P = 0.73$). Thus, thrips that chose the test odor as their first choice were not more likely to choose it as their second choice than thrips that had chosen the control odor as first choice.

Analysis of Volatiles. Comparison of the GC traces from male (upper trace) and female (lower trace) SPME volatile collections (Figure 1) revealed two major peaks in the male trace that were absent from the trace of female volatiles: peak *a* ($t_R = 11.45$ min; abundance = 19.6% by peak area of male-exclusive compounds) and peak *b* ($t_R = 15.11$ min; 67.1%). Comparison of these retention times with those of the larval anal droplet compounds ($t_R = 13.04$ min for decyl acetate; $t_R = 15.45$ min for dodecyl acetate) indicated that they were not the same compounds and could not be the result of contamination with alarm pheromone. Other peaks found in very small amounts in most of the male traces that appeared to be absent from all the female traces were found at $t_R = 7.61$ min, 4.2%; 7.71 min, 0.9%; 7.88 min, 4.1%; 10.61 min, 1.9%; and 10.97 min, 2.3%. The release rate of compound *a* was estimated to be 120 pg male^{-1} hr^{-1}, by comparison of the peak

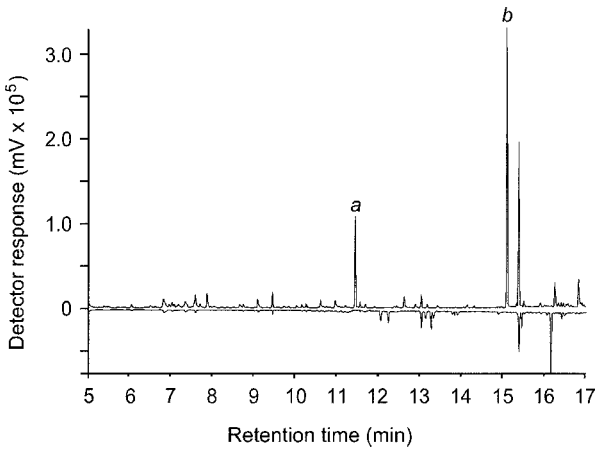

FIG. 1. GC traces of SPME fiber collections of the headspace volatiles collected from mixed-age adult males (upper trace) and mixed-age adult females (inverted lower trace) of *Frankliniella occidentalis*. The major male-specific compounds in the male trace are indicated as peaks *a* and *b*.

area of the unknown with a hydrocarbon standard. The compounds were not found in hexane extracts of males.

DISCUSSION

The results confirm that females in a Y-tube olfactometer are attracted to the odor of males but not to the odor of females, as found by de Kogel and van Deventer (2003), and extend the results to virgin females. The olfactometer preference shown here by virgin females and mixed-age females for 25 males (66–70%) was similar to that of females for 20 or 80 males (68–71%) found by de Kogel and van Deventer (2003). Because adult females can mate within hours of emergence (Terry and Schneider, 1993) and there were many males in the culture, most mixed-age females were likely to have mated. This suggests that the response of females to male odor is not specific to virgins, although this needs to be verified experimentally.

Males were attracted to males, contrary to the findings of de Kogel and van Deventer (2003) for *F. occidentalis* and Milne et al. (2002) for *F. schultzei*. The difference may be because the males in our bioassay were patrolling and fighting, which may affect pheromone production. The entrainment of male-specific volatiles from patrolling and fighting males and the absence of these volatiles from hexane extracts of males collected directly from plants also supports this explanation.

Although the bioassay tested the responses of walking thrips, a flight response is also likely because this would be biologically advantageous for locating aggregation sites and because a flight response by females to males has been shown in another species from the same genus (Milne et al., 2002).

The sternal glands are the most likely source of a male pheromone, but this cannot be verified by dissecting males and comparing extracts from parts of the body because the male-specific compounds appear not to be stored. Abdominal sternal glands or ventral glands occur on adult males in some other insects, such as some true bugs (Schuh and Slater, 1995) and some cockroaches (Sreng, 1984). In the cockroach *Nauphoeta cinerea*, 12 male-specific compounds have been identified from the sternal glands, of which three are the principal active components in the pheromone (Sreng, 1990; Sirugue et al., 1992). Between them, these compounds have roles in attracting females from a distance and in close-range interactions with females and males (Moore, 1997). A male-produced pheromone may also have several behavioral roles in *F. occidentalis*.

The identification of the seven volatile compounds from male *F. occidentalis* is in progress.

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A NOVEL LABORATORY SCREENING BIOASSAY FOR CROP SEEDLING ALLELOPATHY

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Abstract—Crops that control weeds by root exudation of allelochemicals are receiving increased attention, and there are efforts to breed allelopathic cultivars in several crops. The genetic improvement of allelopathic traits is based upon parental germ plasm with high allelopathic activity. Identification of allelopathic germplasm is done in laboratory screening bioassays, but experimental protocols are limited. We developed a fast and reliable laboratory screening bioassay for grain crops that includes dose–response considerations as an integral part of the experimental design. The bioassay was conducted in hydroponic culture, and a range of experiments with 2-(3*H*)-benzoxazolinone (BOA), an allelochemical of several grain crops, was carried out to define the basic protocol. Because of its sensitivity to BOA, *Sinapis alba* L. was selected as the receiver species. BOA affected growth (fresh weight and length of shoot and root), enzyme activities (ascorbate peroxidase, catalase, glutathione S-transferase, peroxidase, phenylalanine ammonia-lyase), and chlorophyll fluorescence, whereby root length was the most reliable response parameter. BOA sensitivity was dependent on nutrients for all parameters measured, and, thus, no nutrients were added. A set of experiments with *Secale cereale* L. and *Triticum aestivum* L. as donor species was carried out to optimize the protocol. Light and pH were eliminated as primary causes for the observed inhibition. The proposed bioassay has several methodological advantages over current bioassays.

Key Words—BOA, 2-(3*H*)-benzoxazolinone, dose–response, hydroponics, laboratory screening, receiver species, response parameter, root exudation, plant-by-plant bioassay.

INTRODUCTION

The discovery that plant-generated chemicals are involved in interference with other plants founded a new concept to manage weeds—crop allelopathy. The

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concept implies the use of crop cultivars with a built-in herbicidal system capable of producing and releasing sufficient amounts of phytotoxic allelochemicals via root exudation that interfere with competing weeds efficiently. Suppression of weeds by root exudation of allelochemicals has proven to be of ecological relevance under field conditions, however, the level is usually not sufficient to provide adequate weed control, or the plant material is not available commercially (Pérez and Ormeño-Núñez, 1993; Dilday et al., 1994, 1998; Chavez et al., 1999; Olofsdotter et al., 1999; Fujii, 2001; Lovelace et al., 2001). Implementation of crop allelopathy is hampered by the lack of highly potent commercial cultivars. Genetic approaches to enhance allelopathic traits in crops have been proposed (Courtois and Olofsdotter, 1998; Duke et al., 2001; Scheffler et al., 2001), but efforts are at a preliminary stage. The basis for genetic improvement of allelopathic traits is the identification of parental germplasm with high phytotoxic activity. If the chemical basis for the effect is known, high-throughput chemical screening of germplasm is probably the favored approach. Since the primary chemical basis is still unknown for most crops, phytotoxic activity is initially assessed by phenotyping. Phenotyping can be categorized as field or laboratory screening, which is further subdivided into aqueous extract and seedling screening (Wu et al., 2001). Various laboratory bioassays have successfully been applied, each developed and adapted to meet specific requirements regarding donor species, target species, test medium, developmental stage, or response parameters (e.g., Putnam and Duke, 1974; Fay and Duke, 1977; Spruell, 1984; Fujii, 1992; Navarez and Olofsdotter, 1996; Hashem and Adkins, 1998; Wu et al., 1998, 2000, 2001). The spectrum of bioassays suggests that seedling phytotoxicity is probably too complex for a single bioassay. For example, establish definite bioassays for different species, eliminate antagonism from nutrients, avoid microbial breakdown of compounds, and test whole plants or plant extracts all require different experimental protocols. Thus, bioassays are not standardized, and the notion that there should be a standardized bioassay is controversial.

Most existing bioassay protocols have methodical limitations when applied to screening large numbers of entries, however, a good mass screening procedure is essential (Courtois and Olofsdotter, 1998). Progress has been made in the development of innovative laboratory methodologies [e.g., the relay seeding technique (Navarez and Olofsdotter, 1996), the equal-compartment-agar method (ECAM) (Wu et al., 1998, 2000), the plant-box method (Fujii, 1992)], but additional screening must be done for most crops before initiating appropriate breeding programs (Wu et al., 2001). For this reason, bioassay-based screening needs to be further developed and its efficiency increased (Courtois and Olofsdotter, 1998).

This study aimed at developing an improved bioassay to screen for seedling allelopathy in grain crops (in particular *Triticum* L. spp. and *Secale cereale* L.). The novelty of the design mainly consists of the incorporation of dose–response considerations and a simultaneous collection of root exudates, thereby promoting

a rapid and simple evaluation. Here, a set of experiments is presented that methodically optimizes each facet of the bioassay protocol, followed by investigations to verify the ease and reliability of the bioassay by eliminating potential confounding factors.

METHODS AND MATERIALS

Bioassay Protocol—Pregermination of Bioassay Species. Seeds of donor species were surface-sterilized in hot water (45°C) for 1 hr (*S. cereale*) or up to 2 hr (*Triticum aestivum* L.), redried for 5 hr at 30°C, and pregerminated in a greenhouse (24/16°C, photoperiod 16:8 L:D, 300 μE/m^{2}s). Pregermination was in plastic pots (11 × 11 × 6 cm) filled with vermiculite (2/3 mm, Gärtnereibedarf Asperg, Germany) for 4–5 d until the first leaf was through the coleoptile. As required, pots were irrigated with tap water. Seeds of receiver species were surface-sterilized [5 min, 70% ethanol; 10 min, 6.5% sodium hypochlorite (13% active chlorine); 30-min rinsing with tap water], wrapped in moisturized filter paper (24 cm in diam., MN 615, Macherey-Nagel), and pregerminated under greenhouse conditions until the cotyledons were completely unfolded.

Bioassay Protocol—Hydroponic Culture. Precultivated seedlings were transferred to aluminum-covered glass beakers (290-ml Sturz-Form, Weck, Germany) covered with polyethylene terephthalate lids (Frischhaltedeckel 80, Weck, Germany) with five holes (each 2 cm in diam.). Membrane pumps (TR E, Schego, Germany) continuously aerated the aqueous test medium (distilled water or nutrient solution). Water losses due to evaporation were adjusted daily.

Bioassay Protocol—Experimental Design. The bioassay was conducted as a dose–response experiment, where allelochemicals were dosed by varying donor plant density. Experiments were carried out using seven different densities ranging from 0 to 45 plants/pot, each with three replicates in a randomized design. At each density, donor plants were arranged in three even portions, and each was placed into one hole of the lid. The density of the receiver species was kept constant at 4 plants/pot arranged in pairs in the two remaining holes of the lid. All dose–response curves were calculated according to Michel et al. (1999) using SPSS® Regression Models and differentiated by means of the lack-of-fit F test ($P = 0.05$).

The Receiver Species. The receiver species was selected by a hydroponic bioassay that investigated the sensitivity of test species to 2-(3H)-benzoxazolinone (BOA, obtained from Fluka). Ten species were prescreened nonreplicated at 100 μg/ml BOA: *Brassica rapa* L. var. *rapa* ssp. *oleifera* (DC.) METZG.; *Brassica rapa* L. var. *esculenta* L.; *Eruca sativa* MILL.; *Lactuca sativa* L. var. *capitata* L.; *Lepidium sativum* L.; *Matricaria inodora* L.; *Raphanus sativus* L. var. *sativus*; *Sinapis alba* L.; *Spinacia oleracea* L.; *Vicia hirsuta* (L.) S.F. GRAY. Dose–response curves were generated for five species using eight concentrations between 0 and 600 μg/ml BOA, each with eight replicates in randomized blocks. Pregerminated

seedlings were individually transferred to aluminum-covered scintillation vials (30 ml) filled with nonaerated, 0.1-fold nutrient solution (Dannel et al., 1998) plus 0.4% (vol.) methanol as solvent for BOA. Controls were performed with methanol only. Test solution was replaced once after 3 d, and the inhibition of shoot fresh weight was evaluated after 6 d. Dose–response curves were calculated by regression analysis.

The Response Parameter—Influence of Nutrients. The influence of nutrient supply on 10 possible response parameters and on their sensitivity to BOA was investigated in a hydroponic bioassay, using *S. alba* as the test plant. Five pregerminated seedlings were transferred to aluminum-covered glass beakers (290 ml) filled with aerated nutrient solution, 0.4% (vol.) methanol, and 0 or 20 μg/ml BOA. The solution was prepared according to Dannel et al. (1998) and tested at a 0.01-, 0.1-, 0.5-, and onefold concentration. Controls were performed at a onefold concentration with methanol only. The test solution was replaced once after 3 d, and the response was recorded after 6 d. Each parameter was evaluated in quadruples as pooled samples of the five *S. alba* plants/pot in a randomized block design. Results were subjected to analysis of variance with multiple comparison (*Tukey*-test, $P = 0.05$) using SPSS® . The following parameters were surveyed: plant growth (length and fresh weight of shoots and roots), specific activity of certain extractable enzymes induced under general stress [ascorbate peroxidase (APX) (Nakano and Asada, 1981), catalase (CAT) (Cakmak and Marschner, 1992), glutathione S-transferase (GST) (Habig et al., 1974), peroxidase (POD) (Hammerschmidt et al., 1982), phenylalanine ammonia-lyase (PAL) (Konstantinidou-Doltsini et al., 1988)], and chlorophyll fluorescence (effective quantum yield; CF) as indicator for a possible impact on photosynthesis (PAM-Fluorometer, Walz, Germany).

The Response Parameter—Detection of Overall Allelopathy. Identification of the parameter best indicating the overall phytotoxic response caused by root exudates of a living donor was examined by means of interference between *S. cereale* cv. Amilo and *S. alba*. The bioassay was conducted in distilled water using the basic protocol. Parameters (except CF) were recorded after 6 d, and corresponding dose–response curves were calculated by regression analysis. The best parameter was selected on the basis of sensitivity (ED_{10} = plant density causing $\pm$10% response), variability [mean standard deviation (SD) per donor density], and simplicity of handling.

Optimizing Preculture of Bioassay Species. The most susceptible developmental stage of the receiver species *S. alba* was determined by evaluating the inhibition of growth by *T. aestivum* cv. Triso at three different preculture periods of *S. alba* (4, 5, and 6 d). The bioassay was conducted in distilled water with a single donor density of 30 plants/pot. Inhibition of root length after 6 d was analyzed by means of an analysis of variance with multiple comparison (*Tukey*-test, $P = 0.05$) using SPSS® , followed by a linear regression analysis with F test ($P = 0.05$).

The feasibility of reducing the single preculture interval of the donor species prior to the bioassay was evaluated with *S. cereale* cv. Amilo applying the basic protocol with *S. alba* as receiver species. Dose–response relationships were generated in distilled water for three different periods of single hydroponic pre-culture (0, 1, and 3 d), and dose–response curves were calculated for the inhibition of the root length of *S. alba* after 6 d.

Optimizing Time of Exposure. The objective of a reduced sampling time was evaluated by comparing the chronology of dose–response curves of *S. cereale* cv. Amilo and *T. aestivum* cv. Pegassos affecting *S. alba*. Dose–response relations were generated in distilled water and inhibition of root length of *S. alba* was recorded daily from 3 to 6 d. The progression of the appropriate curves was compared by means of an F test ($P = 0.05$), and changes in slope B and ED_{50} (plant density causing $\pm 50\%$ response) were evaluated by linear regression analysis with an F test ($P = 0.05$) using SPSS® .

Distinguishing Allelopathy from Competition for Light. Potential effects of differences in light, available under the leaf canopy of donor plants, were investigated by examining the influence of light intensity on the root growth of *S. alba*. Normal greenhouse lighting (16:8 L:D, 300 $\mu E/m^2 s$) was compared to a 50% reduction in light using a hydroponic culture of *S. alba* [5 plants/pot (290 ml) in aerated distilled water]. A shading grid (Gärtnereibedarf Asperg, Germany) provided reduction in light. Root length was measured in triplicates after 6 d, and the means were analyzed by an analysis of variance with multiple comparison (*Tukey*-test, $P = 0.05$) using SPSS® . The actual reduction in light under the conditions of the protocol was evaluated for 13 randomly selected cultivars of *T. aestivum* affecting *S. alba* in distilled water. At the end of the bioassay (6 d), light intensity under the leaf canopy was measured in triplicates at each donor density with a light meter (Panlux electronic, Gossen-Metrawatt, Germany) at the level of the covering lid. Reduction in light was analyzed by means of an analysis of variance with multiple comparison (*Tukey*-test, $P = 0.05$) using SPSS® , followed by a linear regression analysis with an F test ($P = 0.05$). Finally, the cultivar-specific light intensity at a density of 30 plants/pot was linked with the allelopathic activity on *S. alba* (ED_{50}).

Distinguishing Allelopathy from Acidification. Potential effects of acidification of the test medium with time and donor plant density were evaluated in dose–response bioassays using distilled water. The influence of pH on the root growth of *S. alba* [5 plants/pot (290 ml), six replicates] was investigated by adjusting the hydroponic solution to five different pH values (pH 4–6) with 10 M HCl (pH meter, WTW, Germany). Root length was recorded after 6 d. By means of the calculated dose–response curves, pH values that had no statistically significant effect (NOEC, *Tukey*-test, $P = 0.05$) were interpolated, as well as ED_{90} values (pH causing 90% inhibition of root length) according to Streibig et al. (1995). Actual acidification under the conditions of the protocol were evaluated for several randomly selected

cultivars of *T. aestivum* affecting *S. alba*. The pH of the test medium was recorded daily for six cultivars in triplicate at 30 plants/pot, and for 13 cultivars at the end of the bioassay at each donor plant density. Results were subjected to an analysis of variance with multiple comparison (*Tukey*-test, $P = 0.05$) using SPSS®, followed by a nonlinear regression analysis of the sequence of acidification with time and density. Finally, the cultivar-specific pH at the end of the bioassay at a density of 30 plants/pot was linked with the allelopathic activity on *S. alba* (ED_{50}) for 31 cultivars.

RESULTS AND DISCUSSION

The Basic Bioassay Protocol

An essential need in studying crop allelopathy is simulation for natural release of allelochemicals so that chemical interference from living donor plants on living receiver plants can be measured (Olofsdotter et al., 1995; Wu et al., 2000, 2001). Hydroponics is well suited for such whole-plant chemical interaction studies (Einhellig et al., 1982; Liu and Lovett, 1993). It allows easy measurement of various response parameters on roots, which are a sensitive target for most allelochemicals, and facilitates simple, simultaneous collection of exudates. Research in allelopathy is often criticized for ignoring dilution effects caused by varying plant densities or for disregarding dose–response relations (Weidenhamer et al., 1989; Romeo, 2000), so the bioassay was designed as a dose–response experiment with varying donor plant densities. The log-logistic dose–response model (Finney, 1978; Streibig, 1988), which is extensively used for herbicide-based studies (Streibig et al., 1995), proved valid for describing the allelopathic interactions. The same symmetrical sigmoid relationship was observed previously by Wu et al. (2000) evaluating seedling allelopathy of a wheat cultivar against *Lolium rigidum* GAUD using the ECAM method.

The Receiver Species

Ten dicotyledonous species were prescreened to find an easy-to-grow, sensitive, and reliable receiver species. Among other things, the choice was based upon the sensitivity to BOA. *M. inodora* was discarded prior to prescreening because of its unacceptable slow growth rate. Other species varied considerably in their sensitivity to BOA (Table 1). *R. sativus* was almost unaffected by 100 μg/ml BOA and was rejected, despite being easy to grow. The other eight species were more sensitive, with an inhibition of shoot fresh weight varying between $>52\%$ of control (*L. sativa*, *V. hirsuta*) and $<7\%$ (*E. sativa*, *S. alba*). As for sensitivity, all eight species would have been suitable, however, *B. rapa* var. *esculenta*, *S. oleracea*, and *V. hirsuta* were rejected because of slow and/or nonuniform

TABLE 1. INHIBITION OF SHOOT FRESH WEIGHT OF POSSIBLE RECEIVER SPECIES BY 2-(3*H*)-BENZOXAZOLINONE (100 μg/ml)

Species	Shoot fresh weight (% of untreated control)
Eruca sativa	3.2
Sinapis alba	6.8
Spinacia oleracea	12.5
Brassica rapa rapa var. ssp. *oleifera*	26.5
Brassica rapa var. *esculenta*	32.1
Lepidium sativum	37.3
Lactuca sativa	52.6
Vicia hirsuta	55.2
Raphanus sativus	89.9

growth. For the remaining five species, dose–response curves were parallel (B = 2.4), but differed significantly in ED_{50} values (Figure 1). Since parallelism is an indicator of similarity in the mode of action of an active compound (Duke et al., 1983; Jensen and Kudsk, 1988; Streibig, 1988; Kudsk and Streibig, 1993; Streibig et al., 1998), parallel response curves for BOA were expected. Regarding sensitivity to BOA, the dose–response experiment results were similar to the sequence

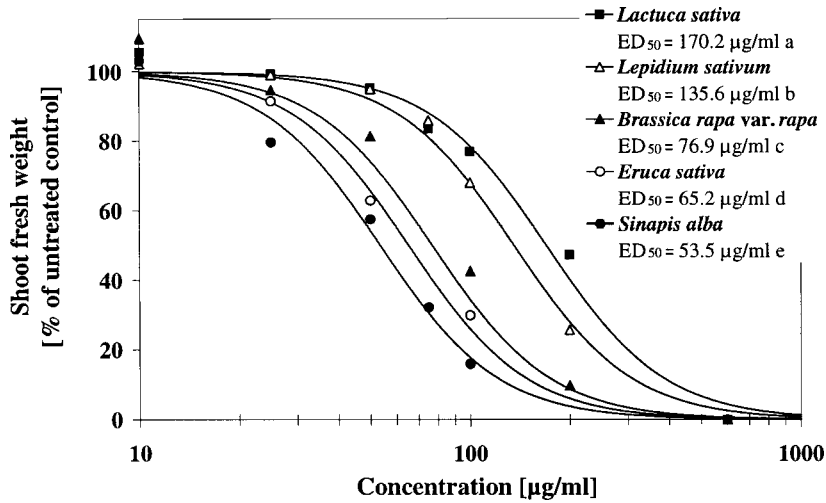

FIG. 1. Sensitivity of five possible receiver species to the allelochemical 2-(3*H*)-benzoxazolinone in hydroponic culture. ED_{50} = plant density causing 50% reduction in shoot fresh weight; small letters indicate significant differences (F test, P = 0.05).

derived from prescreening: most sensitive was *S. alba* with an ED_{50}, more than three times lower than the most insensitive species *L. sativa.* Remarkably, *L. sativa* as well as *L. sativum,* and *R. sativus*, three species often described as being highly sensitive to many allelochemicals (Olofsdotter et al., 1995; Macias et al., 2000), were the most insensitive.

In aqueous solution, the colorless BOA is rapidly degraded by root-colonizing bacteria to the orange-colored 2-amino-(3*H*)-phenoxazin-3-one (APO) (Friebe et al., 1996; Schulz and Wieland, 1999). A corresponding orange coloration of the test solution was visible in all species tested, and the formation of APO was confirmed by an HPLC-MS analysis. Then coloration intensity increased corresponding to BOA concentration and species-specific insensitivity. A visible coloration did not appear for the sensitive *S. alba* until a concentration of 600 μg/ml BOA was used, whereas for the insensitive *L. sativa*, the formation of APO was visible at 10 μg/ml. Since microbial metabolization to APO decreases the phytotoxic influence of BOA (Friebe et al., 1996), the observed species-dependent differences in sensitivity are likely correlated to the ability to degrade the active compound.

The ability to respond to a specific chemical is essential when searching for an indicator species. BOA is a known phytotoxic product of benzoxazinone DIBOA (2,4-dihydroxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one), which is involved in allelopathy of *Triticum* spp. and *S. cereale*. Thus, the receiver species needs to have low microbial metabolization of BOA, as does *S. alba.* In contrast, *L. sativa*, a species successfully used in laboratory screenings of rice (Fujii, 1992; Ebana et al., 2001), could also be used in our bioassay, but its comparably high tolerance to BOA disqualifies it as a good indicator for grain allelopathy. Accordingly, finding a single standard receiver species that is useful in various allelopathic settings seems impossible.

Simplicity of handling and variability of seedling growth was, in contrast to sensitivity, consistent for all species. Pregermination required 5 d for all species, and mean SD for shoot fresh weight was $11.4 \pm 1.8\%$. Therefore, in terms of being easy-to-grow and reliable, any of the five species would qualify as a receiver species, but the higher sensitivity of *S. alba* made it the first choice.

Divergent fundamental perspectives exist concerning receiver species. An ecologically oriented perspective demands a species naturally associated with the allelopathic donor (e.g., Inderjit and Weston, 2000; Romeo, 2000; Wu et al., 2000, 2001). A more pragmatic perspective demands easy-to-grow, sensitive, and reliable indicator species requiring few replications, particularly for initial screening (Olofsdotter et al., 1995). In this study, pragmatism prevailed over ecology.

The Response Parameter

The choice of the parameter measured significantly affects conclusions regarding the interpretation of the effects. Therefore, several potential parameters

were evaluated with regard to their sensitivity to different nutritional conditions and to BOA.

Influence of Nutrients. For ecological relevancy, a nutrient-containing test medium is preferred, but in this case, cultivar- and density-dependent differences in nutrient consumption would confound allelopathy, and an allelopathic interpretation of effects would be valid only if phytotoxicity could be distinguished from competition for nutrients. A parameter indicating a phytotoxic response independent of nutrients would facilitate this. Thus, the influence of decreasing overall nutrient supply on 10 parameters was investigated. Compared to a full-strength nutrient solution (onefold), a 50% decrease (0.5-fold) had no significant influence on any parameter. Further reduction (0.1- to 0.01-fold) influenced some growth parameters and some enzyme activities (Figure 2a and b). Shoot length and fresh weight of *S. alba* were reduced, whereas root length and some leaf enzyme activities were enhanced. The greatest reduction to about 48% of control appeared for shoot fresh weight at 0.01-fold, which also led to an increase to 154% of control in root length. The maximum increase in leaf enzyme activities appeared at 0.1-fold, ranging between 139% of control for APX and 229% for PAL. Therefore, if a nutrient solution were used, a phytotoxic interpretation of the findings recording these parameters would be questionable. In contrast, three parameters proved independent of overall nutrient supply, namely root fresh weight, CF, and leaf CAT activity. If cultivar- and density-dependent differences in nutrient consumption do not exceed the tested range of overall nutrient supply, these parameters appear to qualify for a comparative bioassay in the presence of nutrients.

Sensitivity to BOA. Whether the measured parameters were sensitive to BOA was investigated with 20 μg/ml BOA at onefold nutrient supply. CF and leaf CAT activity showed no significant response (Figure 3a). Both parameters might be appropriate for bioassays in nutrient solution, but, because of their low sensitivity to BOA, they might fail to suggest grain allelopathy and, thus, were rejected. Root fresh weight was the most sensitive to BOA of all growth parameters that were measured.

The essential prerequisite whether the activity of BOA would be constant despite nutritional differences was not met (Figure 3b). Root fresh weight was significantly inhibited at onefold, but the inhibition lessened by increasing nutrient limitation. The same effect occurred for root length; inhibition by BOA was completely reversed by the antagonistic promotion of root length by nutrient limitation. Thus, a nutrient solution might cause a reversion of phytotoxic growth inhibition in the case of high competition for nutrients, i.e., strong nutrient competitors or high plant densities. On the other hand, a nutrient-free solution might fail to detect weak phytotoxic activity due to reduced sensitivity of the receiver. A complementary increase in BOA activity with increasing nutrient limitation appeared for the enzyme activities. For example, although leaf CAT showed no effect at onefold, a limitation gradually enhanced the activity (Figure 3b). Thus, regarding enzyme

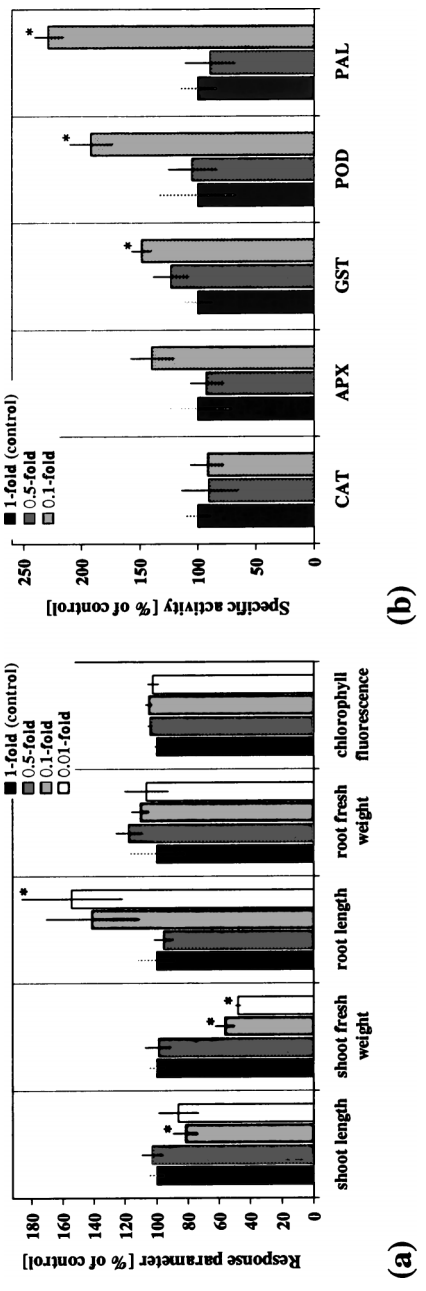

FIG. 2. Influence of nutrient concentration (0.01- to 1-fold) on growth parameters, chlorophyll fluorescence (a) and extractable leaf enzyme activities (b) of *Sinapis alba*. CAT catalase, APX ascorbate peroxidase, GST glutathione S-transferase, POD peroxidase; PAL phenylalanine ammonia-lyase, *significantly different from control (onefold) (Tukey's test, $P = 0.05$).

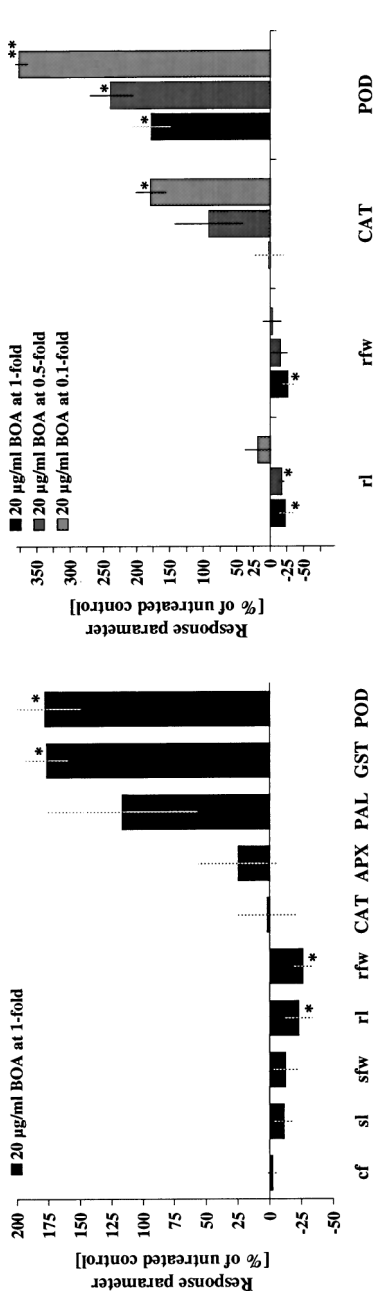

FIG. 3. Effect of 2-(3*H*)-benzoxazolinone (BOA) on growth, chlorophyll fluorescence, and extractable leaf enzyme activities of *Sinapis alba* (a) and the influence of different nutrient concentrations (0.1- to 1-fold) on some of these parameters (b); cf, chlorophyll fluorescence; sl, shoot length; sfw, shoot fresh weight; rl, root length; rfw, root fresh weight; CAT, catalase; APX, ascorbate peroxidase; GST, glutathione S-transferase; POD, peroxidase); PAL, phenylalanine ammonia-lyase; *significantly different from untreated control (0 μg/ml BOA at onefold) (Tukey's test, $P = 0.05$).

activities, competition for nutrients exaggerated the allelopathic effect in contrast to the growth parameters. Whatever parameter was measured, there appeared to be an inseparable overlay of effects. None of the 10 parameters distinguished allelopathy from competition for nutrients.

There are examples where these problems are overcome, e.g., by a continuous monitoring of nutrient contents (Schulz et al., 1994), providing a constant and optimal nutrient supply (Fay and Duke, 1977; Spruell, 1984), or using nonallelopathic control cultivars (Pérez and Ormeño-Núñez, 1991, 1993). These efforts are time-consuming, labor-intensive, and/or costly, and are, therefore, not desirable for high-throughput screening. The criticism that creating artificial conditions in laboratory bioassays may lack ecological relevancy is valid, but the fundamental basis for field crop allelopathy is certainly predicted by a high allelopathic potential in the laboratory, and this can be assessed only by segregation from competition for nutrients. There was no practical way to distinguish allelopathy from nutritional effects and, thus, no nutrients were used in this bioassay, as in most other laboratory screenings (Fujii, 1992; Navarez and Olofsdotter, 1996; Wu et al., 1998, 2000).

Detection of Overall Allelopathy. Almost all parameters tested proved sensitive to BOA and, therefore, suited for assessment of grain allelopathy. However, plant exudates are a mixture of mostly lower-molecular-weight substances and their metabolites. Overall chemical interference via root exudation will probably be based upon a combination of phytotoxic metabolites and will influence a wide range of metabolic pathways. The parameters investigated here are mainly indirect measurements of several physiological processes and should be suitable for an overall estimate of allelochemical potential. On the basis of the interference of *S. cereale* cv. Amilo on *S. alba*, we evaluated which parameter would be most responsive and the easiest to measure. There was considerable variability among responses (Figure 4). Shoot fresh weight and length were unaffected at any donor density tested. An absence of shoot growth response was observed previously for *L. rigidum* exposed to *T. aestivum* (Wu et al., 2000). It is certainly possible that a higher donor density, a prolonged length of exposure, or more effective cultivars might result in significant effects, but within the present protocol, shoot growth was insensitive and, therefore, rejected. Root fresh weight and length were significantly inhibited, and dose–response curves were identical with an average ED_{10} of 4.9 plants/pot (Table 2). Although SD was lower for the measurement of the root fresh weight, root length was preferred because it was easier to measure.

In contrast to the inhibition of root growth, extractable root enzyme activities were variable and density-dependent, being both stimulatory and inhibitory. ED_{10} values ranged from 1.2 plants/pot for PAL to 12.2 plants/pot for APX (Table 2). Thus, PAL, followed by CAT, was the most sensitive, while POD, GST, and APX were equally or less sensitive than root growth. Compared to the influence of BOA

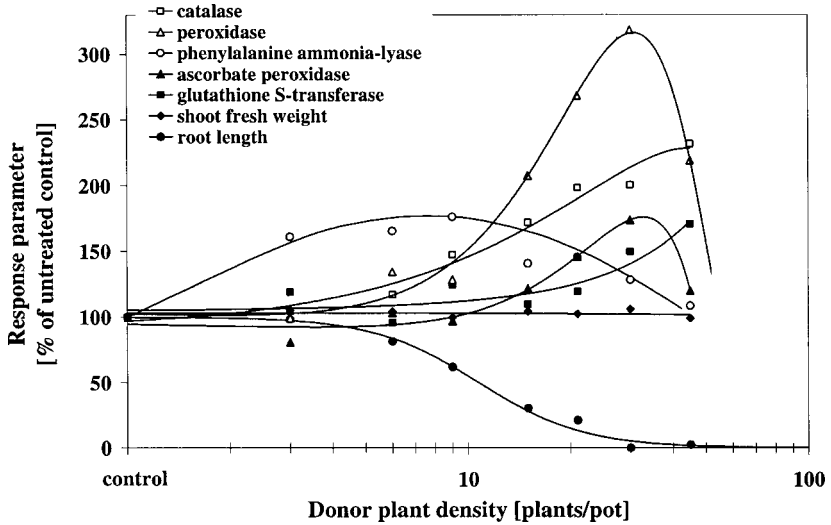

FIG. 4. Dose–response relations of seven possible test parameters (extractable root enzyme activities, shoot fresh weight, root length) on the interference of *Secale cereale* cv. Amilo with *Sinapis alba*.

on extractable leaf enzyme activities of *S. alba* (Figure 3), the sequence of sensitivity changed. Different sequences of sensitivity were observed previously for the same enzymes when *Helianthus annuus* L. and *Lycopersicon esculentum* MILL. were exposed to various herbicides (Greiner, 2001). Consequently, it appears that a suitable enzymatic parameter for a specific objective has to be selected on a case-to-case basis, depending on species, plant organ, and active compound(s). This specificity seems crucial regarding the screening of different species and cultivars potentially exuding different allelochemicals.

TABLE 2. DETECTION OF ALLELOPATHIC IMPACT OF *Secale cereale cv.* AMILO ON *Sinapis alba* RESPONSE PARAMETERS

Parameter	PAL[a]	CAT[a]	Root fresh weight	Root length	POD[a]	GST[a]	APX[a]	Shoot length	Shoot fresh weight
ED_{10}[b] (plants/pot)	1.2	3.2	4.9	4.9	4.9	7.5	12.2	>45	>45
SD[c] (%)	30.1	71.0	12.7	16.4	30.1	22.1	21.9	6.6	8.5
Handling[d]	−	−	+/−	++	−	−	−	++	++

[a] Extractable root enzyme activity: PAL phenylalanine ammonia-lyase, CAT catalase, POD peroxidase, GST glutathione S-transferase, APX ascorbate peroxidase.
[b] ED_{10} = donor plant density causing ±10% response.
[c] SD = mean standard deviation per donor density.
[d] handling: −, difficult; +, good; ++, easy.

A further critical aspect regarding enzyme activities as response parameters is concentration, since similar responses can result from two different dosages of allelochemicals. Therefore, enzymes are unsuitable for comparative bioassays at a single given donor density. The three major screening protocols are usually conducted at a single donor density (Fujii, 1992; Navarez and Olofsdotter, 1996; Wu et al., 1998, 2000), thus, for these protocols, the enzymes tested here would be inappropriate. Although enzymes are suitable for a dose–response design and highly sensitive, their activities are also highly specific, highly variable, and labor-intensive to perform. For these reasons, enzymes were rejected as response parameters for low-replicated high-throughput screening of different species. Thus, the final decision about the ultimate parameter seemed clear: root length, the same parameter that has been used in the other three major screening bioassays currently in use (Fujii, 1992; Navarez and Olofsdotter, 1996; Wu et al., 1998, 2000).

Optimizing Preculture of Receiver Species

Lowering the time of preculture of *S. alba* to increase sensitivity proved effective. The inhibition of root length by *T. aestivum* cv. Triso was significant at any time of preculture, but linearly decreased from 70% of control for 4 d of preculture to 43% for 6 d (Figure 5a). Younger plants were more sensitive, and control plants showed a higher growth rate in distilled water, reaching a final root length of 30 cm, compared to 23 cm for 6 d of preculture. A greater amplitude of response seemed desirable and, thus, *S. alba* was exposed to donor plants after 4 d of pre-culture. At this time, plants had an average root length of 4.9 ± 0.5 cm, the minimum length required for treatment in the hydroponic system and the baseline for the dose–response curves.

Comparison of the linear regression lines revealed that root length of control plants decreased with a slope of -3.8, whereas it increased in cocultivation with a slope of 2.0. Previous investigations on photosynthesis of two growth stages of *Beta vulgaris* L. revealed a similar change in slope for a treatment with glufosinate, while changes in light, a major factor of competition, simply lead to a parallel shift of linear regression lines (Kobusch, 2003). Thus, if competition had been the primary cause for the observed inhibition in our bioassay, a parallel shift would have occurred, but instead the slope significantly changed. The fact that the observed response was contradictiory to the predicted effects of competition provides evidence for a noncompetitive, allelopathic interaction.

Optimizing Preculture of Donor Species

To allow an accumulation of allelochemicals to active concentrations, the addition of the receiver species to the test system is usually delayed (Navarez and Olofsdotter, 1996; Wu et al., 2000). However, a brief time delay improves the utility

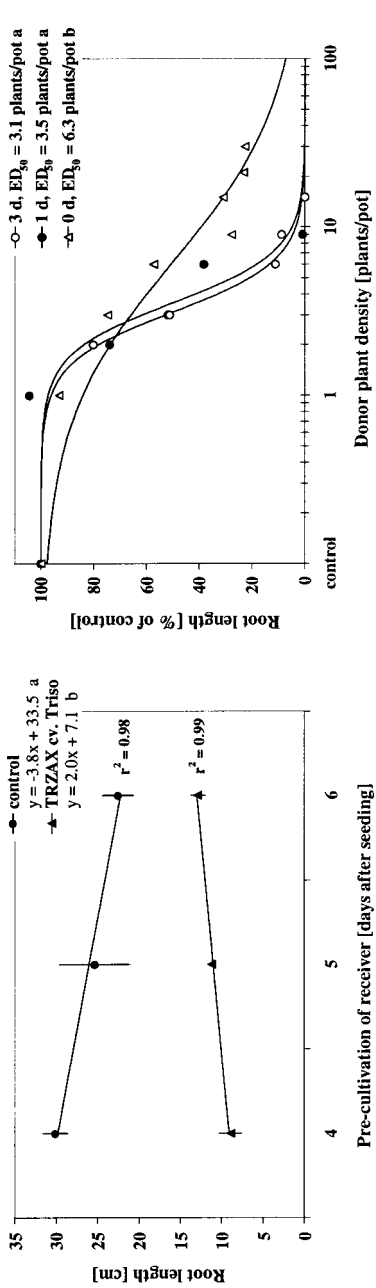

FIG. 5. Inhibition of root length of *Sinapis alba* by the interference with *Triticum aestivum* (TRZAX) cv. Triso (30 plants/pot) at three different pre-culture periods of *S. alba* (a) and *Secale cereale* cv. Amilo at three different single hydroponic preculture periods of *S. cereale* (0, 1, and 3 d) (b). ED_{50} = plant density causing 50% reduction in root length; small letters indicate significant differences (F test, $P = 0.05$).

of the bioassay. For an accurate description of a dose–response relationship, the dose range should cover complete inhibition (Seefeldt et al., 1995; Michel et al., 1999). Preliminary tests with our protocol showed that a delay of 3 d was sufficient to achieve complete inhibition of root growth of *S. alba* at high donor densities. The feasibility of reducing the time of delay and still attaining complete inhibition was investigated by comparing the dose–response curves of *S. cereale* cv. Amilo at three different delay periods. No significant differences were observed between a delay of 1 and 3 d ($B = 2.9$, mean $ED_{50} = 3.3$ plants/pot) (Figure 5b). The absence of delay failed to inhibit root length of *S. alba* completely at any density, resulting in a nonparallel dose–response curve ($B = 0.9$, $ED_{50} = 6.3$ plants/pot). Because an increase in plant density from 21 to 30 plants/pot was ineffective in increasing the effect, a complete inhibition of root growth apparently cannot be achieved in the absence of a delay. A stronger phytotoxic cultivar might be able to do so, but screening determines the assessment of a wide range of allelopathic potential, including weak phytotoxic cultivars. To reduce the risk of obtaining inaccurate dose–response curves, a 1-d delay of cocultivation was selected. In comparison to 7 d of delay applied in the relay seeding technique (Navarez and Olofsdotter, 1996) and the ECAM method (Wu et al., 1998), the current protocol allows a marked shortening of delay.

Optimizing Time of Exposure

The possibility of shortening sampling time (6 d) was investigated for two cultivars. The results confirmed that *S. cereale* cv. Amilo was more inhibiting than *T. aestivum* cv. Pegassos at any time. Dose–response curves were always parallel, i.e., the chronological proportion of the ED_{50} among cultivars was constant, but ED_{50} differed significantly. On average, the ED_{50} values of cv. Pegassos were 2.4 times higher than those of cv. Amilo. Despite a constant proportion, the regression parameters (ED_{50}, slope B) changed with time. However, the time-dependent correlation between ED_{50} and B was identical for both cultivars and showed a linear reciprocal proportionality (Figure 6). The results suggested that a dose–response curve can change with time, but conclusions regarding comparative classification of phytotoxic activity were independent of sampling time. Since, the observed changes in relative dose–response relations were not significant, a decrease of sampling time seemed legitimate. Nevertheless, the length of exposure was kept at 6 d, as the quality of the regression, indicated by the size of confidence intervals of estimates, and, thus, the deterioration of the accuracy of estimating the allelopathic potential went hand in hand with the reduction of the sampling time. Compared to the 10-d sampling time of the relay seeding technique (Navarez and Olofsdotter, 1996) and the ECAM method (Wu et al., 2000), as well as the 2–12 weeks of the plant-box method (Fujii, 1992; Hashem and Adkins, 1998), the current protocol allows for a marked shortening of exposure.

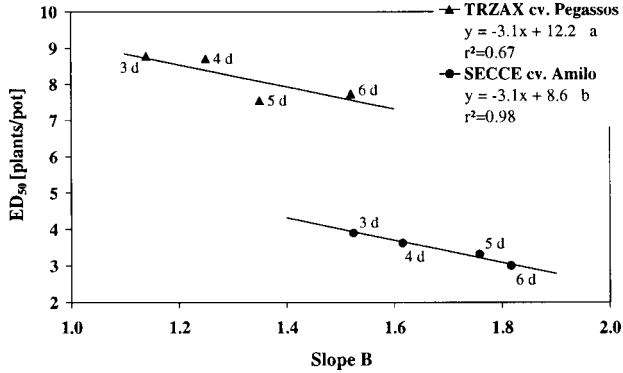

FIG. 6. Dose–response relations of *Secale cereale* (SECCE) cv. Amilo and *Triticum aestivum* (TRZAX) cv. Pegassos as a function of the length of exposure. Interdependency of changes in ED_{50} and slope B with increasing sampling time (3–6 d). ED_{50} = plant density causing 50% reduction in root length; small letters indicate significant differences (F test, $P = 0.05$).

Distinguishing Allelopathy from Competition

This far, we have attributed the observed inhibition to phytotoxicity, and the essential question of whether the observed effects were actually due to allelochemicals was disregarded. Research in allelopathy is often criticized for the lack of elimination of other mechanisms of interference (Romeo, 2000). In particular, possible effects of competition or abiotic factors are often ignored, but distinguishing or separating phytotoxicity from other mechanisms of plant–plant interaction is essential before justifying an allelopathic conclusion. In the present bioassay, two other aspects might confound allelopathy: light and pH.

Light. Root length of *S. alba* was insensitive to an artificial reduction in light of about 50% of normal greenhouse illumination. Measuring the actual reduction of light intensity under the leaf canopy of donor plants revealed a significant decrease at the end of the bioassay depending on donor density and cultivar. Nevertheless, for each of the 13 cultivars tested, the light intensity under the leaf canopy remained far above the level where there was any effect on the root length observed earlier (Figure 7a). Furthermore, the cultivar-specific decrease in light at 6 d and a density of 30 plants/pot was not linked with allelopathic potential on *S. alba* (ED_{50}) (Figure 7b). Thus, existing cultivar- and density-dependent differences in light under the leaf canopy are not a primary cause for the observed inhibition of the root growth of *S. alba.*

pH. S. alba grows best in lime-rich soils and only tolerates a range of soil pH of about 4.5–8.2 (Duke, 1983). Thus, a reduction in pH with increasing accumulation of root exudates with time and density might make pH a determining factor. The

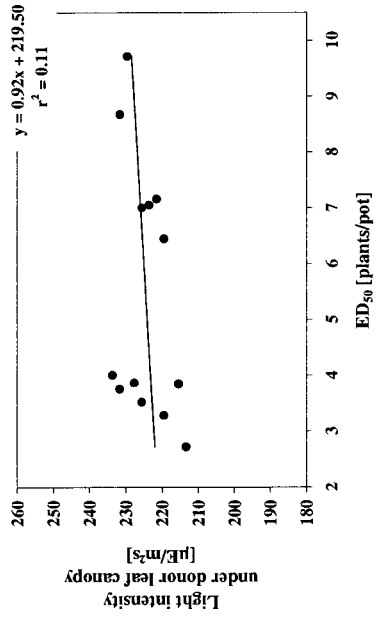

FIG. 7. Decrease in light under the plant leaf canopy of 13 randomly selected cultivars of *Triticum aestivum* with increasing plant density (a) and the interdependency between cultivar-specific light intensity at the end of the bioassay at 30 plants/pot with allelopathic potential on *Sinapis alba* (b). ED_{50} = plant density causing 50% reduction in root length of *S. alba*.

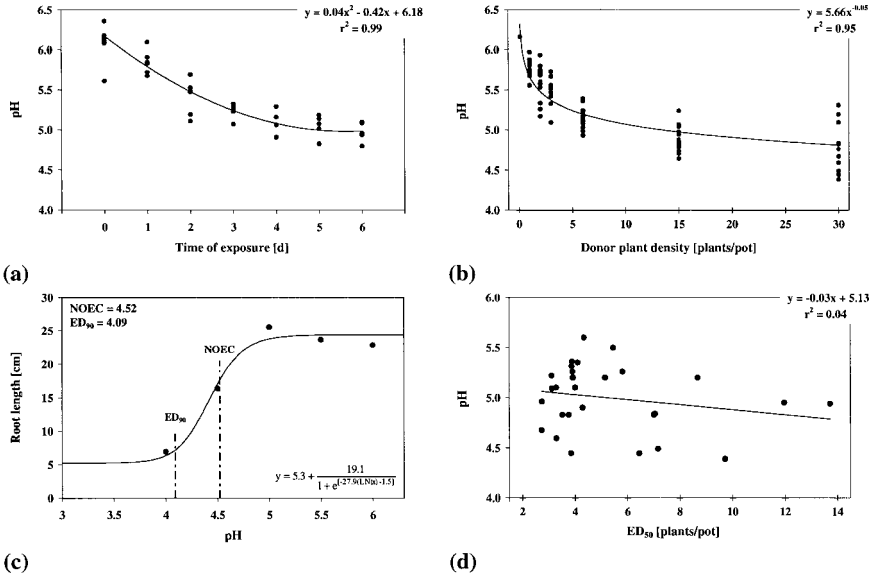

FIG. 8. Influence of pH in hydroponic culture. (a) Acidification of test medium by root exudates of *Triticum aestivum* with time of exposure (six cultivars, 30 plants/pot). (b) Acidification with plant density of *T. aestivum* at 6 d (13 cultivars). (c) Influence of artificial acidification on the root length of *Sinapis alba*. (d) Correlation between cultivar-specific pH at the end of the bioassay at 30 plants/pot and ED_{50} (31 cultivars of *T. aestivum*). NOEC = pH value of no observable effect (Tukey's test, $P = 0.05$); ED_x = plant density causing x% reduction in root length of *S. alba*.

pH in the bioassay gradually decreased with sampling time from a mean pH of 6.1 at 0 d to pH 5.0 at 6 d (six cultivars, 30 plants/pot). Acidification was already significant at 1 d, and cultivar-specific acidification differed significantly (Figure 8a). Furthermore, a significant decrease with donor density was apparent at the end of the bioassay, with a mean pH of 6.2 at 0 plants/pot to pH 4.8 at 30 plants/pot. Cultivar-specific differences in acidification with donor density were also significant (Figure 8b). Therefore, the relevancy of acidification was investigated. An artificial acidification of the hydroponic culture had no statistically significant effect on the root length until a pH of 4.52 (NOEC), which corresponds with the lower limit of the pH range for the natural occurrence of *S. alba* (Duke, 1983). Further acidification increasingly inhibited the root length down to a pH value of 4.09 (ED_{90}), where almost no further growth took place (Figure 8c). Consequently, at 79% of the 19 cultivars tested, acidification remained above the NOEC at any time and density. For these cultivars, the observed inhibition could not be caused by pH. At four cultivars, acidification undershot the NOEC at the end of the bioassay

at the highest density, but the pH remained far above the ED_{90}. A contribution of pH effects could, thus, be possible for some cultivars at 30 plants/pot, but a sole causality for the observed complete inhibition at this density was not substantiated. Finally, the absence of a linkage between cultivar-specific acidification (pH at 6 d and 30 plants/pot) and allelopathic activity on *S. alba* (ED_{50}) made a contribution of pH unlikely (Figure 8d). This, along with the elimination of light as a confounding factor, suggests that phytotoxicity is operative.

The Final Bioassay Protocol

In summary the final design of the laboratory bioassay is divided into four steps: pre-culture, bioassay, evaluation, and correlation (Figure 9). The *pre-culture* of the donor plants is done in vermiculite for 4–5 d, and for 1 d in hydroponic culture. The receiver plants are germinated wrapped in filter paper starting 4 d prior to the bioassay. The *bioassay* is done as a mixed hydroponic culture in aerated distilled water with a sampling time of 6 d. Four plants of *S. alba* are cocultured with seven different donor plant densities (0–30 plants/pot) in triplicates in a completely randomized design. The allelopathic potential is *evaluated* as inhibition of root length of *S. alba*, and ED_{50} values are calculated by means of nonlinear regression analysis. Complementary, root exudates are collected and the amount of allelochemicals quantified via chemical analysis. The *correlation* of the cultivar-specific allelopathic potential (ED_{50}) to the allelochemical content in root exudates provides an indication of the primary chemical basis for allelopathy.

Compared to existing methodologies that screen for potential seedling allelopathy under laboratory conditions, this method features: applicability to all grain crops and to a wide range of receiver species; the possibility of measuring several response parameters on roots or shoots; suitability to testing early stages of development accomplished by a low time of pre-culture (4-d receiver, 4 to 5-d donor), a short time of delay (1 d), and a short sampling time ($\geq$3 d); the possibility of testing various donor densities, from individual plants to 30 plants/pot without risking major influence from changes in light and pH; simple, nondestructive collection of root exudates at any time of the bioassay; and easy handling and low costs of material. The assay is fast and simple, and, thus, suitable for high-throughput screening. Additionally, the successful incorporation of dose–response considerations as part of the protocol makes it appropriate for new approaches and complex studies on seedling allelopathy, and the obtained ED_{50} values probably generate a better estimation of the allelopathic potential than a single-point assessment.

On the other hand, the bioassay has real and potential limitations. For example, the use of distilled water may constrict the applicability to certain species, especially dicotyledons, and older developmental stages. The hydroponic culture demands an almost daily adjustment of water losses, and sterile conditions are difficult to create. The maximum donor density is spacial limited to approximately

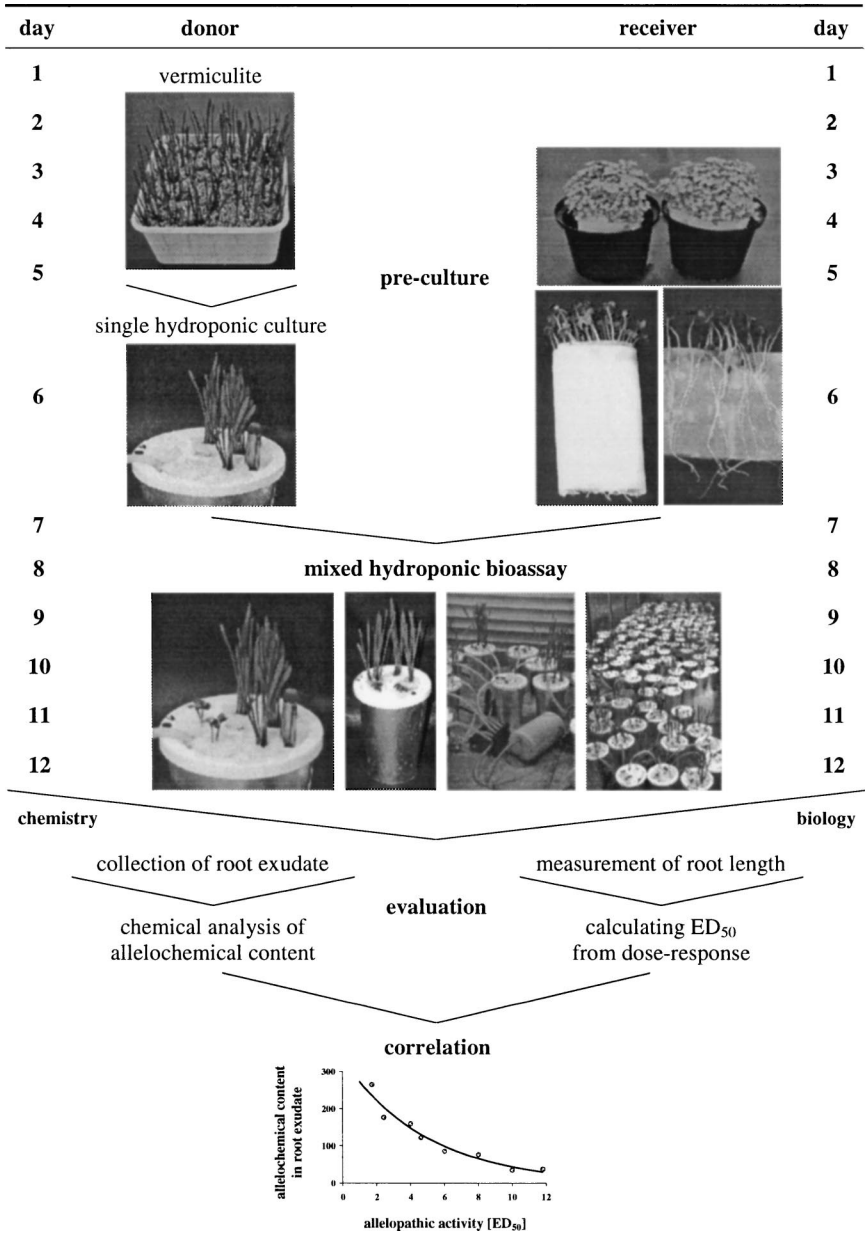

FIG. 9. The final design of the laboratory bioassay for allelopathic activity of grain crops. ED_{50} = plant density causing 50% reduction in root length of the receiver species.

45 plants/pot (290 ml), and pH effects cannot be precluded at this density. The arrangement of plants on the covering lid is not completely arbitrary. Furthermore, the dose–response design requires high rates of germination of donor plants especially for the higher densities, which can be a problem for poorly germinating cultivars and/or small quantities of available seeds. The required lead-time and space of a dose–response design is naturally higher than for a single-point assessment with only one donor density. Moreover, with regard to a high allelopathic potential, the approximation to or the undershooting of an ED_{50} value of 1 plant/pot may hamper the calculation of dose–response relations and the accuracy of discrimination among strong phytotoxic cultivars. However, the screening will prove if ED_{50} values of $\leq$1 plant/pot are realistic. If this is the case, the problem may be solved in terms of a dilution by using larger pots.

The bioassay is not claimed to be the best possible approach to screen for potential seedling allelopathy, because "there is no perfect bioassay. . ." (Leather and Einhellig, 1985). The assay is, however, reliable, simple, and fast, and facilitates high-throughput screening. The next step will be its application to the characterization of the allelopathic potential of a wide range of grain crop cultivars that should prove whether the constraints are significant.

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MANIPULATION OF ROOT HAIR DEVELOPMENT AND SORGOLEONE PRODUCTION IN SORGHUM SEEDLINGS

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Abstract—Sorghum (*Sorghum bicolor*) roots exude a potent bioherbicide—sorgoleone. Previous work indicates that sorgoleone is produced in living root hairs. We have developed a mist system that resulted in abundant production of root hairs exuding sorgoleone and a mat system that significantly inhibited root hair development and consequently sorgoleone production. Applying Ag^+ (an ethylene action inhibitor) at 1.2 mM to the seedlings grown in the mist system also inhibited root hair formation and elongation. Hypoxic conditions in the mist system did not result in the inhibition of root hair growth as compared to the standard air atmosphere (20.8% O_2). Applying ethephon (an ethylene-releasing agent) at 0.031 mM to the roots of seedlings grown in the mat system with water running at 1 ml/min reversed the inhibition of root hair development by water movement. These results indicate that either water movement or ethylene can be utilized to manipulate root hair development and sorgoleone production in sorghum seedlings. It is hypothesized that water movement reduced the local ethylene concentration on the root surface and consequently inhibited root hair development of sorghum seedlings grown in the mat system.

Key Words—Root hair, *Sorghum bicolor*, sorgoleone, ethylene, oxygen, ethephon, silver thiosulphate, allelopathy, hypoxic condition.

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INTRODUCTION

Allelopathy offers great potential for alternative weed control through the release of allelochemicals toxic to competing vegetation. Various researchers have proposed genetically engineering allelochemical production into plants for the development of transgenic crops that produce their own defense against weeds (Gressel, 2000; Duke et al., 2001). Certain plant species possess a potent weed-suppressing ability, e.g., sorghum [*Sorghum bicolor* (L.) Moench], winter rye (*Secale cereale* L.), winter wheat (*Triticum aestivum* L.), and others (Weston, 1996). Sorghum roots exude biologically-active hydrophobic substances, one major component of which is known as sorgoleone, characterized as 2-hydroxy-5-methoxy-3-[(8′*Z*, 11′*Z*)-8′, 11′, 14′-pentadecatriene]-*p*-benzoquinone (Chang et al., 1986; Netzly and Butler, 1986; Nimbal et al., 1996). Sorgoleone is toxic to both small-seeded broadleaf and grass weeds at concentrations as low as 10 μM in root uptake studies (Einhellig and Souza, 1992; Nimbal et al., 1996). It is an inhibitor of both photosynthetic (Einhellig et al., 1993; Gonzalez et al., 1997; Rimando et al., 1998) and mitochondrial electron transport (Rasmussen et al., 1992; Einhellig, 1995; Czarnota, 2001). In addition, Meazza et al. (2002) reported that sorgoleone strongly inhibited hydroxyphenylpyruvate dioxygenase (HPPD) at concentrations lower than that of juglone, a napthaquinone produced by black walnut (*Juglans nigra* L.). HPPD is the novel target site for a new family of triketone herbicides. Inhibition of this enzyme disrupts the biosynthesis of carotenoids and results in foliar bleaching (loss of chlorophyll), which is also observed in sorgoleone-treated seedlings (Nimbal et al., 1996).

Recent work in our laboratory suggested that sorgoleone is produced only in living root hairs (Czarnota et al., 2001). Root hairs are tubular extensions of epidermal cells that have their origin either in any protoderm cells or in specialized protoderm cells called trichoblasts (Cormack, 1949; Row and Reeder, 1957; Ridge, 1995; Peterson and Farquhar, 1996; Pemberton et al., 2001; Ryan et al., 2001). Root hairs contribute as much as 77% to the total root surface area of cultivated crops, forming the major point of contact between the plant and the rhizosphere. (Parker et al., 2000). Root hairs play important roles in plant life processes, including uptake of water and nutrients, production of substances that mediate plant–microbial associations, regulation of plant growth, and determination of microbial community structure in the plant rhizosphere (Fan et al., 2001; Grierson et al., 2001; Michael, 2001; Inderjit and Weston, 2003).

Normal root hair development encompasses four events: hair initiation, swelling, transition to tip growth, and tip growth (Parker et al., 2000). Root hair development is either favored or restricted by certain physiological factors. Increasing the temperature of the rhizosphere, especially above the optimum for the plant, often leads to enhanced root hair development and shorter, thicker roots. However, the influence of aeration on root hairs is unclear (Michael, 2001). When a growing root meets a physical obstruction, root hair formation is increased (Roberts, 1993;

Michael, 2001). Dark-grown wild-type *Arabidopsis* seedlings, which produce little ethylene, are largely hairless. However, exogenous treatment of dark-grown *Arabidopsis* plants with either ethylene or 1-aminocyclopropane-1-carboxylic acid restores the development of root hairs in cells overlying the anticlinal cortical cell walls (Cao et al., 1999). Genes involved in ethylene signal transduction influence root hair elongation. Mutations in *ethylene receptor 1* (*ETR1*) and *EIN2* (which encodes for a metal transporter protein for ethylene signal transduction) inhibit root hair elongation. Mutations in *constitutive triple response 1* (*CTR1*), the negative regulator of ethylene signaling, promote root hair elongation. Therefore, it is hypothesized that ethylene plays an important role in root hair initiation and elongation (Dolan, 2001).

Ethylene is a potent inhibitor of cortical cell division, an effect that can be counteracted by applying silver ions (Ag^+), which block the ethylene signal transduction pathway (Heidstra et al., 1997; Pereira-Netto, 2001). The rhizosphere environment influences ethylene production within the roots and gas exchange at the root surface. If gas exchange is enhanced, the removal of ethylene will be accelerated, thereby limiting its function in hair formation (Michael, 2001). The final step in the ethylene biosynthetic pathway (the conversion of ACC to ethylene) requires molecular oxygen, as shown in *Arabidopsis* silique tissue grown under subambient oxygen (2.5 or 5.5%) conditions where no subsequent ethylene was detected (Ramonell et al., 2002).

In this study, we attempted to manipulate root hair development and sorgoleone production in sorghum seedlings by physiological regulation of ethylene production or action for the purpose of establishing a basis for analysis of gene expression associated with sorgoleone production in sorghum root hairs. Specifically, the effects of hypoxic conditions, Ag^+ (an inhibitor of ethylene action), ethephon (an ethylene releasing agent), and movement of air/water surrounding developing roots on root hair formation in sorghum (*S. bicolor* × *S. sudanense*) SX17 were examined.

METHODS AND MATERIALS

Plant Material and Growth Conditions. Sorghum (*S. bicolor* × *S. sudanense*) SX17 seeds (Monsanto Company, St. Louis, MO) were utilized in all laboratory studies. Seed was rinsed three times in tap water and surface sterilized with 15% (v/v) bleach (CLOROX®) containing 5.25% sodium hypochlorite for 10 min. After three washes in purified water (RiOs 16 system, Millipore Corporation, Bedford, MA), seed was germinated for 60 hr at room temperature (24 ± 1°C) in the dark using two different growth systems:

1. The mat growth system is described in Figure 1A. A glass plate (20 × 50 cm; 0.4 cm thick) was slanted at approximately 4°. Two layers of cheese cloth (20 × 20 cm) were spread over the glass plate, and 200 seeds were

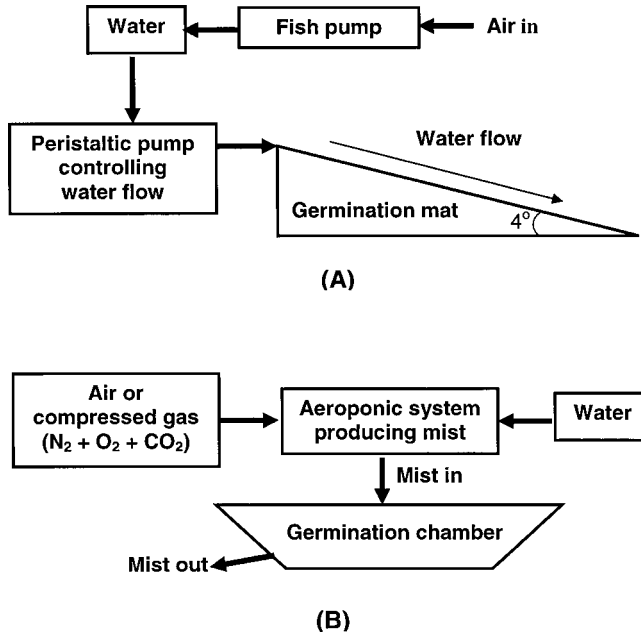

FIG. 1. Diagram of two systems for regulating root hair development in sorghum seedlings. (A) The mat system. The germination mat consists of a glass plate sloping 4°, two layers of cheese cloth over the plate, 200 seeds on the cheese cloth, a layer of clear polyethylene covering the seeds, a glass plate over the plastic sheet. Water was applied to the cheese cloth. (B) The mist system. Seeds were germinated inside a black plastic germination chamber under flowing moist mist supplied by the aeroponic system.

spread evenly on the cheese cloth. A layer of clear 2-mil polyethylene sheeting (Lab Safety Supply Inc., Janesville, WI) was used to cover the seeds. Finally, a matching glass plate (20 × 50 cm; 0.4 cm thick) was placed on top of the plastic sheet. To evaluate the influence of the flow rate of water on root hair development, the water supply was controlled by a peristaltic pump (2232 MicroPerpex S, LKB BROMMA, Sweden) adjusted to provide a flow rate of 0.04, 0.07, 0.2, 0.4, 1.0, or 4.0 ml/min. For an even water distribution, the water was pumped onto a cheese cloth strip (6-layer, 1 cm wide) along the upper edge of the mat. To examine the effect of exogenous ethylene on root hair development in the mat system, ethephon (Fisher Scientific, Pittsburgh, PA) was added to the water supply at a concentration of 0.031 mM at 24 hr following seeding. After another 24 hr, the ethephon solution was replaced by a fresh preparation of the same concentration.

2. The mist growth system is described in Figure 1B. Two hundred seeds were germinated on 400 cm^2 of paper towel spread over an aluminum screen

slanted at approximately 4° in a plastic chamber exposed to a flowing mist provided by an aeroponic system (NUTRI-MISTER™ 0100, Waterford Equipment Company, Big Flats, NY) with an ultrasonic probe. To examine the effect of mist flow on root hair development, the mist flow rate in the aeroponic system was set at 15, 68, or 136 l/min. These settings influenced the relative amount of moisture produced, but not relative humidity, which was maintained at 100%. To examine the effect of oxygen on root hair development, seedlings were exposed to one of four oxygen concentrations (0.2, 2.0, 10.5, or 20.8% O_2). Compressed O_2, CO_2, and N_2 (Airgas East, Inc., Ithaca, NY) were used to obtain gas mixtures containing 0.2, 2.0, 10.5, or 20.8% O_2 plus 0.035% CO_2 in N_2 by controlling flow rate using flow meters (Bel-Art Products, Pequannock, NJ). The gas mixture was utilized by the aeroponic system to keep the mist flowing. Controlled atmospheres were monitored every 12 hr by gas chromatography (Fisher GAS PARTITIONER, model 1200, Fisher Scientific, Pittsburgh, PA) using a thermal conductivity detector and two different columns (165 × 3 mm, packed with 80/100 mesh Porapak Q; 279 × 5 mm, packed with 60/80 mesh molecular sieve 13X). The oven temperature was 90°C, and the injector temperature was 135°C. The carrier gas was helium with a flow rate of 30 ml/min. To examine the effect of Ag^+ (an inhibitor of ethylene action) on root hair development, water was replaced by silver thiosulphate (STS) solution for generating mist after 24 hr of germination. STS solutions were prepared by mixing $AgNO_3$ with $Na_2S_2O_3$ in a 1:4 (w/v) molar concentration ratio (Kamaluddin and Zwiazek, 2002).

Measurements of Root Hairs. After seed was germinated for 60 hr, 10 seedlings were randomly selected from each experimental run. A 1-cm root segment was excised from each seedling beginning at 0.5 cm behind the root tip. Root segments were fixed in 70% ethanol, placed on microscope slides (Corning Incorporated, Corning, NY), and covered by a glass cover. Three digital images were taken for each root segment from the ends and the middle using an Olympus BX-50 light microscope equipped with Differential Interference Contrast optics (Olympus America Inc., Melville, NY), a high resolution PentaMax cooled CCD camera (Princeton Instruments, Trenton, NJ), and MetaMorph software package (Version 4.5r4, Universal Imaging Corporation, Downingtown, PA). Root hair length and number present in the middle region (684 μm) along the root length in each digital image were determined using the ruler tool in the AlphaEase (v5.5) software (Alpha Innotech Corporation, San Leandro, CA).

Two independent experimental runs were performed for each treatment, with each run giving statistically similar results. For all experiments, the means of two independent runs were combined. Data presented are the mean values recorded from 20 seedlings (±95% confidence interval). One-way ANOVA with a Tukey's *post hoc* test was performed to separate means.

High Performance Liquid Chromatography (HPLC) Analysis. Root exudates were extracted as described by Czarnota et al. (2001). Briefly, 2 g samples of fresh roots were dipped in 100 ml of methylene chloride (acidified with 0.25% glacial acetic acid) for ~2 min. After the roots were removed from the solvent, the extract was filtered through an Acrodisc® CR 25 mm syringe filter with a 0.2-μm PTFE membrane (Pall Corporation, Ann Arbor, MI). The filtrate was concentrated to 2 ml with a rotoevaporator (Büchi ROTAVAPOR R-111, Brinkmann Instruments, Inc., Westbury, NY) at 40°C. Concentrated root exudates were transferred to preweighed vials, and dried under N_2 gas. Root exudates were reconstituted with methylene chloride (acidified with 0.25% glacial acetic acid) to a concentration of 1 mg exudates/ml. HPLC analysis of root exudates was performed as described by Czarnota et al. (2001). Briefly, 15 μl of root exudates (1 mg/ml in methylene chloride acidified with 0.25% glacial acetic acid) were subjected to HPLC analysis (Waters 2695 Separations Module, Waters Corporation, Milford, MA) using a Waters NovaPak C18 column (150 × 3.9 mm). The mobile phase was eluted at 2 ml/min according to the following program: 0–15 min, 45% A (2.5% acetic acid in water) + 55% B (acetonitrile); 15–22 min, linear gradient from 55 to 100% B; 22–25 min, 100% B; 25–26 min, linear gradient from 100 to 55% B; and 26–30 min, 45% A + 55% B. The components of the root exudates were detected at 280 nm with a Waters 996 photodiode array detector (Waters Corporation, Milford, MA), and identified by using a purified sorghum root exudate sample of which several components were chemically characterized (Rimando et al., 1998; Czarnota et al., 2001).

RESULTS

Effect of Air/Water Flow on Root Hair Development. Variations in the rate of air flow from 15 to 136 l/min did not significantly influence root hair density and length when seedlings were produced in the mist system. In comparison, root hair density and length were affected by the variations in the flow rate of water from 0.00 to 4.00 ml/min in the mat growth system, with a larger effect on root hair density than on root hair length (Figure 2). In this system, water flow at 0.07 ml/min or higher resulted in significant reductions in root hair density and length, while primary root length was not significantly inhibited (data not shown). When water flow rate was 0.4 ml/min or higher, the roots produced in the mat system were predominantly hairless (Figures 2A and 3F).

Effect of Hypoxic Condition on Root Hair Development. In the mist growth system, there was no significant difference in root hair density between hypoxic (0.2–10.5% O_2) and ambient oxygen conditions (20.8%) (Figure 4A). Treatment with 2% O_2 significantly increased the root hair length as compared to the ambient oxygen concentration (20.8% O_2), while two other subambient oxygen concentrations (0.2 and 10.5% O_2) did not result in significant differences in root

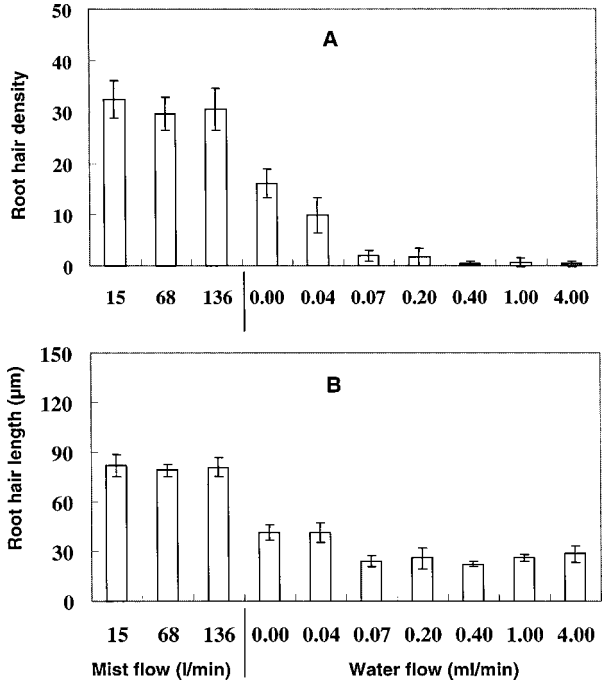

FIG. 2. Influence of water and air movement on root hair development. Sorghum seeds were germinated for 60 hr using the mat or mist system as illustrated in Figure 1. Root hair density was measured as root hair number per 684 μm root segment. Values shown represent the mean of 20 seedlings $\pm$95% confidence interval.

hair length (Figure 4B). The elongation of the primary roots was markedly inhibited by 0.2% O_2 compared to the standard air atmosphere (data not shown). These data indicate that reducing oxygen concentration in the mist system can not be utilized to efficiently inhibit root hair development, while maintaining normal root elongation.

Effect of Ag^+ on Root Hair Development. An application of 0.4 mM Ag^+ in the mist system significantly reduced root hair density and root hair length while primary root length was not significantly reduced as compared to the control (Figure 5). Increasing Ag^+ concentration resulted in further reduction in root hair density and root hair length, and Ag^+ generally had a larger effect on root hair length than on root hair density (Figure 5A and B). Root hair development was arrested at the swelling stage by a treatment with 2.00 mM Ag^+ (Figure 3D). However, under mist containing 2.00 mM Ag^+, primary root growth was also markedly inhibited (Figure 5C). These data indicate that in comparison to modified O_2 atmospheres, Ag^+ can efficiently inhibit root hair elongation, but can not cause

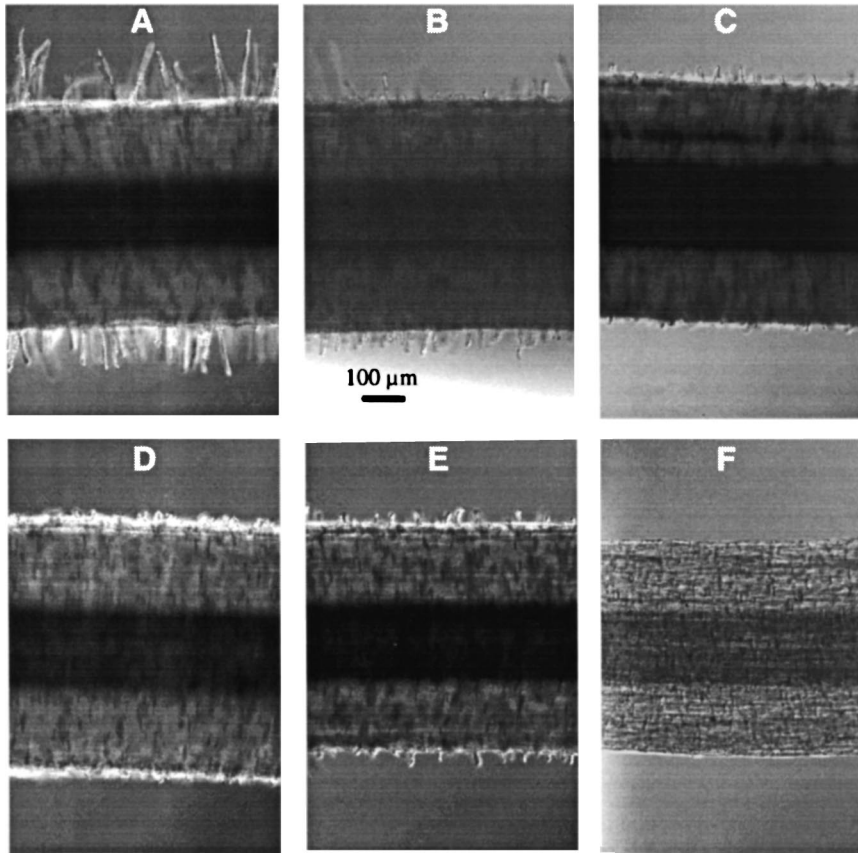

FIG. 3. Light micrographs of sorghum roots showing the effects of different environmental conditions. (A–D) Seed was germinated for 24 hr under water mist, followed by 36 hr under water mist containing 0, 0.4, 1.2, and 2.0 mM Ag^+, respectively. (E) Seed was germinated for 60 hr in the mat system with water movement at 1.0 ml/min. Ethephon was added to the water supply at 0.031 mM 24 hr following seeding. After another 24 hr, the ethephon solution was replaced by a fresh preparation of the same concentration. (F) Seed was germinated for 60 hr in the mat system with water movement at $\geq$0.4 ml/min.

complete inhibition of root hair formation even when the Ag^+ concentration is increased to a level inhibitory to primary root growth. Therefore, Ag^+ can not be utilized to efficiently inhibit root hair initiation, while maintaining normal root elongation. It is reasonable to suggest that ethylene promotes root hair development in the seedlings of sorghum SX17, but is not the only positive regulator of this phenomenon.

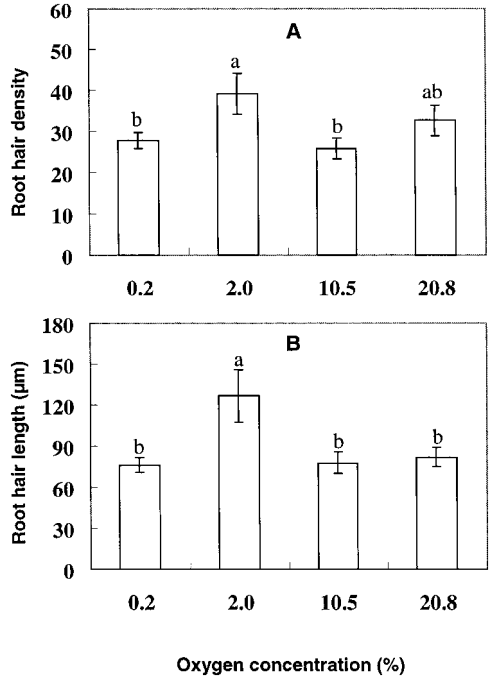

FIG. 4. Influence of hypoxic condition on root hair development in the mist system. Sorghum seeds were germinated in a standard air atmosphere (20.8% O_2) for 24 hr, followed by 36 hr of the indicated modified oxygen treatment. Root hair density was measured as root hair number per 684 μm root segment. Values shown represent the mean of 20 seedlings $\pm$95% confidence interval. Different letters are used to indicate means that differ significantly ($P < 0.05$).

Effect of Ethephon on Root Hair Development. An application of ethephon at 0.031 mM to the water supply, flowing at 1 ml/min, significantly increased root hair density and root hair length as compared to the treatment with no ethephon added. There was no significant difference in root hair density and length between the treatment with no water flow and the treatment with a 0.031 mM ethephon solution moving at 1 ml/min (Figure 6). These data indicate that ethephon can reverse the inhibition of root hair development by water movement in the mat system.

Root Exudation. Sorghum root exudates were collected from seedlings grown in the mist system. The root exudate contained at least five related compounds including sorgoleone, as identified using a purified sorghum root exudate sample in which several components were chemically characterized (Rimando et al., 1998; Czarnota, 2001). In comparison, the roots of seedlings grown under the mat system

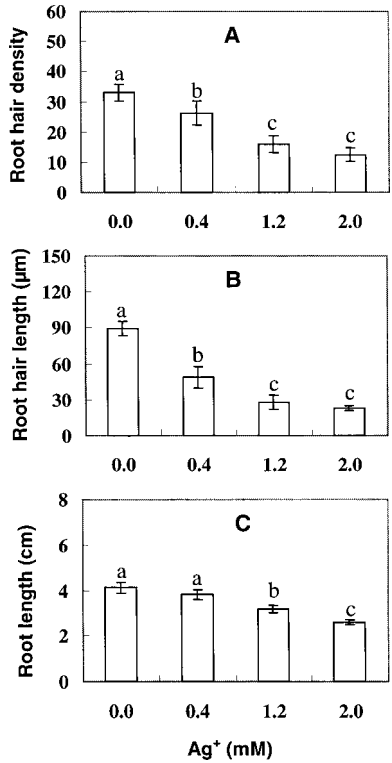

FIG. 5. Influence of Ag^+ on root hair development in the mist system. Sorghum seeds were germinated for 24 hr under mist, followed by 36 hr under water mist supplemented with Ag^+ (0, 0.4, 1.2, or 2.0 mM). Root hair density was measured as root hair number per 684 μm root segment. Values shown represent the mean of 20 seedlings $\pm$95% confidence interval. Different letters are used to indicate means that differ significantly ($P < 0.05$).

maintaining a high water flow rate of 15 ml/min did not produce any sorgoleone in the root extracts (Figure 7).

DISCUSSION

Recent work in our laboratory has identified the site of sorgoleone synthesis as the sorghum root hairs (Czarnota et al., 2001). Successful manipulation of root hair production can facilitate analysis of gene expression associated with sorgoleone production in sorghum root hairs. We have established two growth systems that produce significant differences in root hair development, while minimizing

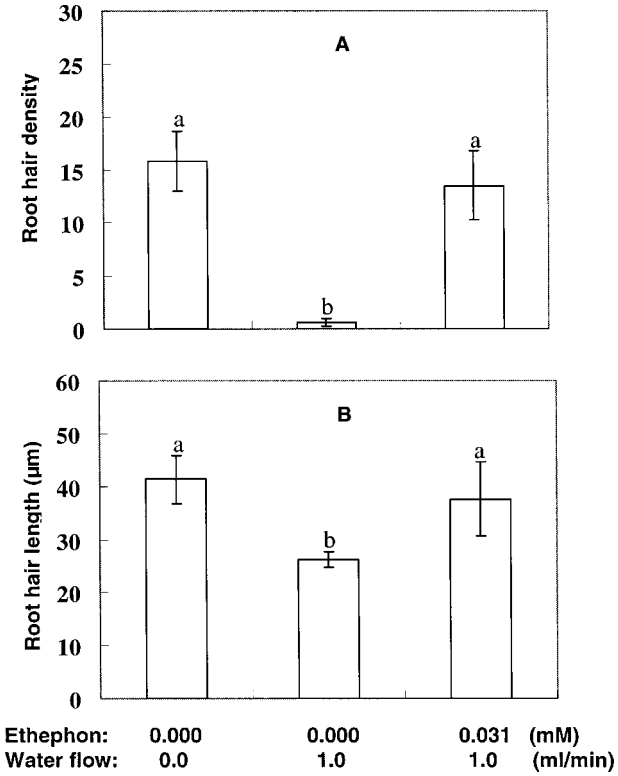

FIG. 6. Effect of ethephon on root hair development in the mat system in relation to water flow rate. Root hair density was measured as root hair number per 684 μm root segment. Values shown represent the mean of 20 seedlings $\pm$95% confidence interval. Different letters are used to indicate means that differ significantly ($P < 0.05$).

differences in primary root growth. Sorghum seedlings grown in our mist system with air flow at 15 l/min produced abundant root hairs exuding sorgoleone, whereas those grown in our mat system with water flow at $\geq$0.4 ml/min produced predominantly hairless roots which subsequently did not exude sorgoleone (Figures 2 and 7).

As compared with ecotype Columbia, the ethylene-overproducing *Arabidopsis* mutants *eto1-1*, *eto2*, and *eto3* exhibited increased numbers and lengths of root hairs. The ethylene-insensitive mutants *etr1-1*, *ein2-1*, and *ein3-1* exhibited decreased root hair lengths, but normal root hair densities (Wubben et al., 2001). These findings support the assumption that ethylene promotes root hair development, but is not the only positive regulator of this phenomenon. This assumption is also supported by our experiments. For example, applying the ethylene inhibitor

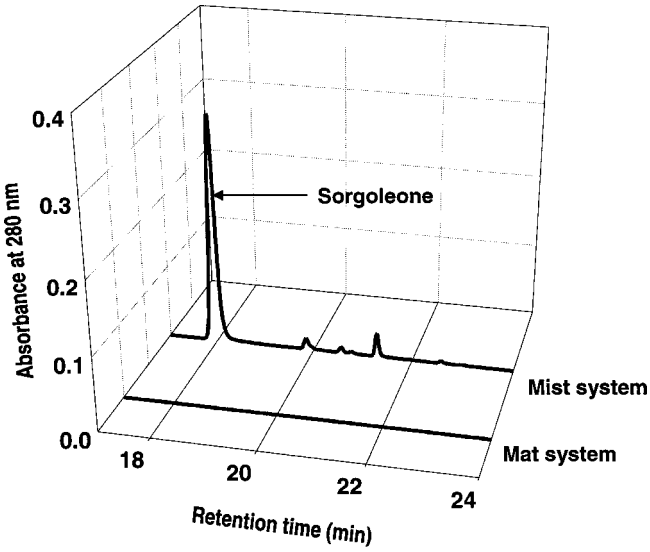

FIG. 7. HPLC profile of root exudates produced by the roots of sorghum SX17 seedlings. The seeds were germinated for 60 hr using the mat system with water flow at 15 ml/min and mist system with air flow at 15 l/min as illustrated in Figure 1.

Ag^+ to sorghum seedlings grown in the mist system resulted in significant inhibition of root hair initiation and elongation. However, even when Ag^+ concentration was increased to a level that was inhibitory to primary root growth, root hair initiation was not completely suppressed (Figures 3D and 5). Recently, it was reported that endogenous auxin plays a complementary role for root hair development in the absence of an ethylene response in *Arabidopsis* (Rahman et al., 2002).

The final step in the ethylene biosynthetic pathway (the conversion of ACC to ethylene) requires the presence of oxygen. It was recently reported that ethylene was not detected in *Arabidopsis* silique tissue produced under subambient oxygen (2.5 or 5.5%) conditions (Ramonell et al., 2002). However, in our studies, the reduction of O_2 supply in the mist system did not inhibit root hair growth. In contrast, a moderate hypoxic condition (2.0% O_2) enhanced root hair growth in comparison to standard atmosphere conditions (Figure 4). Brailsford et al. (1993) also reported that 3 or 5% O_2 increased ethylene production in the primary roots of 72-hr-old seedlings of *Z. mays* cv. LG11 as compared to 1.0, 12.5, or 20.8% O_2, and no ethylene production was detected in the complete absence of oxygen. We hypothesize that in our studies, 2% O_2 resulted in an enhanced ethylene production in sorghum roots as compared to 0.2, 10.5, or 20.8% O_2 and, thus, increased root hair length.

Root hair initiation and elongation of sorghum seedlings grown in the mat system were significantly inhibited as compared to those grown in the mist system (Figure 2). In the rhizosphere, an increase in gas exchange at the root surfaces accelerates removal of ethylene and consequently limits its function in root hair formation (Michael, 2001). Similarly, it can be expected that water running over the roots can accelerate the movement of endogenous ethylene away from the root surfaces, thus inhibiting root hair formation. This speculation is supported by our experiments with the ethylene-releasing agent ethephon. Applying ethephon at 0.031 mM (4.5 ppm) reversed the inhibition of root hair development by water flow at 1 ml/min in the mat system (Figures 3E, F, and 6).

The presence of living root hairs clearly influences the amount of sorgoleone that is exuded by living sorghum roots. Our studies indicate that ethylene influences root hair formation and subsequently sorgoleone production. In soil, ethylene accumulation is influenced by various factors such as microorganisms, humus, total nitrogen, water tensions, and oxygen supply (Zechmeister-Boltenstern and Nikodim, 1999). Therefore, it is possible that in field practice we can manipulate these soil factors to optimize ethylene concentration in the rhizosphere and consequently enhance root hair development and sorgoleone production in sorghum cover crops in an effort to develop crop-based weed management system. For analysis of differential gene expression associated with root hair development and sorgoleone production in sorghum, our mist system with air flow at 15 l/min can be used to produce sorghum roots with abundant roots hairs exuding sorgoleone, while our mat system with water movement at $\geq$0.4 ml/min can be used to produce predominantly hairless roots which do not exude sorgoleone.

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POTENTIAL TOXIC EFFECT ON AQUATIC FAUNA BY THE DWARF SHRUB *Empetrum hermaphroditum*

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Abstract—The common evergreen dwarf shrub *Empetrum hermaphroditum* has influence on the functioning of boreal terrestrial ecosystems in northern Sweden. The negative effects of *E. hermaphroditum* are partly attributed to the production of the dihydrostilbene, batatasin-III, which is released from leaves and litter by rain and snowmelt. In this study, we investigated whether batatasin-III is carried by runoff into streams and lakes during the snowmelt period and whether it is also potentially hazardous to aquatic fauna. Sampling of water from streams and a lake for which the surrounding terrestrial vegetation is dominated by *E. hermaphroditum* was done during the snowmelt period in May 1993 and in 1998, and analyzed for batatasin-III. Using 24- and 48-hr standard toxicity tests, we analyzed toxicity to brown trout (*Salmo trutta*) alevins and juvenile water fleas (*Daphnia magna*). Toxicity (proportion of dead individuals) to trout was tested at pH 6.5 and compared with that of a phenol within a range of concentrations. In the toxicity (proportion of immobilized individuals) test on *D. magna*, the interactive effect of pH (pH 5.5–7.0) was included. Concentration of batatasin-III was generally higher in 1998 than in 1993 and showed peak levels during snowmelt. Concentration in ephemeral runnels > the lake > streams running through clear-cuts dominated by *E. hermaphroditum* > control streams lacking adjacent *E. hermaphroditum* vegetation. The maximum concentration of batatasin-III found was 1.06 mg l^{-1}. The proportion of dead yolk sac alevins

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increased significantly ($P < 0.001$) with increasing concentrations of batatasin-III and time of exposure. After 24 hr, EC_{50} was 10 mg l^{-1}. It was 2 mg l^{-1} after 48 hr. The effect of phenol was negligible, indicating a specific phytotoxic effect of the bibenzyl structure of batatasin-III. The proportion of mobile *D. magna* became significantly smaller ($P < 0.001$) with increasing concentrations of batatasin-III, with decreasing pH, and with increasing exposure time. EC_{50} varied between 7 and 17 mg l^{-1} at pH 5.5 and 7.0, respectively. After 24 hr EC_{50} decreased and was 2.5 at pH 5.5 and 12 mg l^{-1} at pH 7.0. The levels of batatasin-III found in the field samples were below the lowest EC_{50} in acute toxicity tests. However, in view of the interactive effect of pH and exposure time, this study suggests that this stable plant metabolite may impose a lethal effect on the aquatic fauna in small streams.

Key Words—*Empetrum hermaphroditum* Hagerup, toxicity test, batatasin-III, yolk-sac alevins, brown trout, *Daphnia magna*, pH effect.

INTRODUCTION

Modern forestry has exploited most of the north European boreal forests and drastically altered properties of several natural ecosystems including biodiversity (Gamlin, 1988; Östlund et al., 1997). The environmental consequences of clear-felling and the subsequent planting of tree seedlings in forests that are naturally regenerated after wildfire are poorly understood (Zackrisson et al., 1996; Linder et al., 1997). In northern Scandinavia, effective wildfire suppression has increased the occurrence of the common evergreen dwarf shrub *Empetrum hermaphroditum* in late successional forests. After clear-felling, numerous areas that are poorly regenerated tend to become dominated by *E. hermaphroditum* (Sarvas, 1950; Haapasaari, 1988; Nilsson, 1992). The dominance is partly attributable to the production of high levels of water-soluble phenolics, and in particular to the dihydrostilbene, batatasin-III ($C_{15}H_{16}O_3$), which is leached from green leaves and litter (Odén et al., 1992; Wallstedt et al., 1997; Nilsson et al., 1998). In natural forests, batatasin-III constitutes up to 6% of the green leaf dry weight (Wallstedt et al., 1997) and may, in some cases, even exceed 11% (C. Gallet, unpublished data). Batatasin-III is comparatively stable in the soil and can accumulate to sufficient levels to influence biological activity (Wallstedt et al., 1997; Gallet et al., 1999). Several field and laboratory experiments and detailed chemical analyses of leaf material and soil have confirmed that this plant species has inhibitory effects on both plants and soil microbes (Nilsson, 1994; Wallstedt et al., 1997, 2000; Wardle et al., 1997, 1998; Nilsson et al., 1998, 2000) and may be toxic to other organisms as well. The cellular mechanism by which batatasin-III operates is not fully known, but Wallstedt et al. (2001) recently found that batatasin-III inhibited proton pumping in isolated plant plasma membrane vesicles, but only slightly inhibited ATP hydrolytic activity, suggesting that it disturbs membrane integrity and fundamental processes such as ion uptake and H-ATP activity.

The snowmelt period appears critical in determining the high occurrence of batatasin-III in soil water solution during spring. The compound can accumulate in high concentrations because of low temperatures and anaerobic conditions in wetted humus. Accumulation also results from leaching from decomposing litter. This probably creates inferior conditions for soil microbial degradation (cf. Nilsson and Zackrisson, 1992; Wallstedt et al., 1997). Because of ground ice during spring, snowmelt is prevented from draining into the ground, and potentially this allows batatasin-III to drain into small streams. Small streams are important reproduction and nursery habitats for several aquatic organisms such as brown trout (*Salmo trutta*) and zooplankton (Elliott, 1974). Brown trout spawn in the fall, and the eggs hatch in February–April, depending on latitude. The alevins hatch with large yolk sacs and remain in the gravel for 2–4 wk until the yolk sac is absorbed, after which they emerge from the gravel for exogenous feeding at the peak abundance of the zooplankton drift (Braum, 1978). There are several small potential trout stream habitats with poor or nonexistent fish populations. The poor production may depend on several single factors or the combined effects of physical barriers such as dams, changes imposed by logging, and lethal water chemistry such as acid shocks at snowmelt (Roni et al., 2002). As the period of brown trout emergence coincides with the peak levels of batatasin-III found in forest soil humus, this compound has an hazardous effect on these aquatic organisms. In addition, acid shocks may have synergistic effects with temporary elevated level of batatasin-III, as the toxic effect of phenols increases at lower pH (Horner et al., 1988). A large number of tropical plants are known to have poisonous properties on aquatic organisms (Kuhnert, 1991; Thomson, 1991). However, no studies to date have reported on the toxicity of plant species on aquatic animals in the boreal forest.

In view of the increasing area converted by *E. hermaphroditum* in European northern boreal forests in recent years (Nilsson, 1992), as well as the general phytotoxic properties of this plant species, the aim of the present study was to (1) investigate whether batatasin-III produced by *E. hermaphroditum* can be transported during early spring into aquatic forest ecosystems and (2) whether batatasin-III leached from surrounding terrestrial vegetation can be a potential threat to the aquatic fauna, e.g., fish and water fleas.

METHODS AND MATERIAL

Water Sampling and Analysis of Batatasin-III. To quantify natural levels of batatasin-III, water samples were collected from two areas: (i) a natural forest drainage basin at Ledvattnet, northern Sweden (65°30′ N, 18°30′ E, 450 m.a.s.l.) and (ii) a National Forest Mountain Reserve at Akkanålke (65°30′ N, 19°00′ E, 650 m.a.s.l.) (Figure 1). The Ledvattnet area is dominated by *E. hermaphroditum*, following clear-cutting in the 1950s of a mixed Scots pine (*Pinus sylvestris*) and

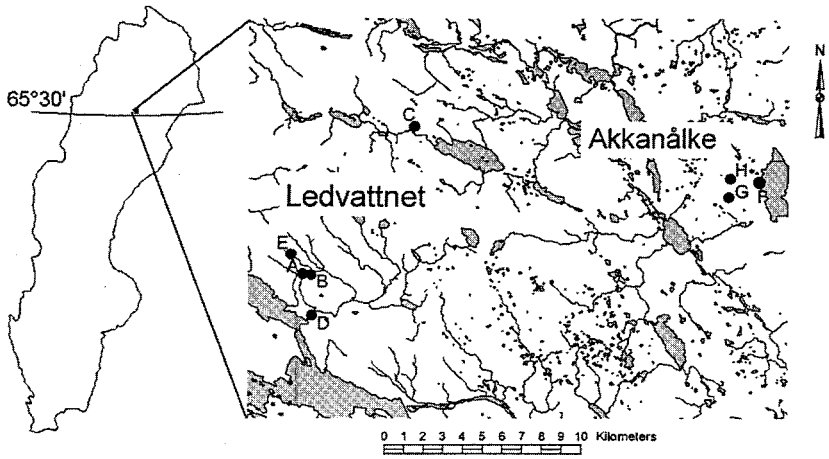

FIG. 1. Map of the field site in northern Sweden. Locations for water sampling are marked with filled circles.

Norway spruce (*Picea abies*) forest. Water was sampled from two ephemeral runnels (A and B) carrying snowmelt, which had flooded the ground vegetation (dominated by *E. hermaphroditum*), and from two streams (C and D) that drained clear-cut areas with ground vegetation dominated by *E. hermaphroditum* (Figure 1). One tributary stream (E), which mainly drained a Norway spruce forest dominated by *Vaccinium myrtillus*, was sampled as a control (Figure 1). At Akkanålke, water samples were collected from a small (about 1 ha) lake (F) with abundant shore vegetation of *E. hermaphroditum*, with no previous history of clear-cutting and from two nearby sites (G and H) with ephemeral snowmelt runnels draining through the *E. hermaphroditum* vegetation close to this lake (Figure 1). At each of these eight sites, duplicate 5-l water samples were collected at regular intervals in 1993 and in 1998 between the peak of the snowmelt period and the fall. Sampling and storage were made in PE containers. Data are presented as mean values among replicate samples.

The concentration of batatasin-III was analyzed for each water sample. Chromatographic separation was performed by using a Waters 600 Controller, equipped with a diode array detector Waters 996 and Millenium software. A 250 $\times$ 4.6 mm column filled with microBondapak C18 10 μm, was used for this procedure. Analysis was carried out on water samples (injection volumes: 20 μl) with a linear gradient at a flow rate of 1.5 ml min^{-1}, from 0 to 60% of B (0.5% of acetic acid in acetonitrile) in A (0.5% of acetic acid in distilled water) for 30 min, with 15 min of reequilibration between samples. Quantification was performed by comparison with calibration curves with pure batatasin obtained from Cyclopss Biochemical

Corporation, Salt Lake City, UT, USA. The pH of water samples was simultaneously monitored.

Toxicity Test on Brown Trout Alevins. Two weeks posthatched yolk sac alevins from a mixed batch of sea trout from River Vindeln stock raised in a local hatchery were used in the experiment. After transportation to the laboratory, 10 alevins were put into each of 64 net-mesh boxes (size $7 \times 10 \times 15$ cm) and submerged into a 10-l aquarium with a flow through (1 l min^{-1}) 6°C tap water. The tap water is a soft, high-quality, sand-bed filtered, nonchlorinated water with pH 8 (± 0.5), a temperature of 6°C, and an oxygen level close to saturation. The fish were acclimatized for 2 d before the start of the experiment.

Acute 24 and 48 hr toxicity tests were used to evaluate the effects of different concentrations of batatasin-III on brown trout alevins. As the *E. hermaphroditum* metabolite is a phenol, and high concentrations of phenolics have a general inhibiting effect there is a possibility that the effect of batatasin-III is nonspecific (cf. Escher et al., 1996). Therefore, a phenol (C_6H_5OH) was used as a control. Each compound was first dissolved in ethanol and then diluted in MilliQ water to obtain a concentration series of 22.5, 15.0, 12.0, 10.5, 9.0, 7.5, 5.25, 3.75, 2.25, and 0 mg l^{-1} of batatasin-III and of 22.5, 15.0, 10.5, 5.25, 2.25, and 0 mg l^{-1} of phenol, respectively. Each of the test media was replicated four times and buffered to pH 6.5, using a standardized EPA method with NaOH, KH_2PO_4, and HCl for constituting buffers.

The exposures started by submerging one net-mesh box with 10 individuals into each of the sixty-four 1-l aquariums filled with 0.7-l buffered solution. Each aquarium was aerated with standard aquarium pumps. Test aquaria were kept in a dark climate chamber (6°C), and the number of dead alevins was counted and removed after 24 and 48 hr. Oxygen content in each test treatment was checked after 24 and 48 hr using an oxygenometer (Microprocessor Oximeter WTW), and the pH was checked with a standard pH-meter.

Toxicity Test and Interference with pH on Daphnia magna. A standard acute toxicity test (cf. Berglind et al., 1985) was used to test toxicity of batatasin-III on *D. magna*, representing zooplankton, the main prey of emerging brown trout (Braum, 1978). *D. magna* was kept in a room with a photoperiod of 16L:8D and a temperature of 20 ± 0.5°C during both culture and exposure. Before the experiment, *D. magna* were kept in 300 ml flasks containing a culture media, according to Klüttgen et al. (1994) supplemented with the cultured algae *Selenestrum* and *Chlamydomonas*, yeast, serofyll extract, and vitamins following Berglind et al. (1985). As the carbonate in the culture media has a buffering capacity, the construction of media of lower and stabilized pH was achieved by mixing the culture media with tap water (1:5) then aerating it. The pH of the media was buffered to 7.0, 6.5, and 6.0, respectively, using a standardized EPA method (EPA, 1975) for constructing different pHs. A modification of the EPA method with a higher

proportion of acid to base (0.12 ml NaOH/70.0 ml KH_2PO_4) to 2.0 l of water was used to buffer the media to pH 5.5.

For this experiment, a two-factor experimental design encompassing exposure of *D. magna* to culture media (see above) amended with 20, 16, 12.8, 10,2, 8.2, 6.6, 5.2, 4.2, and 0 mg l^{-1} of dissolved batatasin-III (see Methods and Materials section) at four different pH levels was set up. Each experimental unit consisted of a 50-ml flask and was replicated four times. Female juveniles, maximum 24-hr old, *Dapnia magna* ($N = 7$–14) were transferred to each flask. After 24 and 48 hr, the number of immobilized individuals was counted. Alkalinity, conductivity, mg oxygen, and pH were measured before and at each sampling occasion.

A probit model (LIMDEP) was used to analyze the effect of exposure concentration, exposure time, and pH. Survival curves were fitted to the data, and the proportion of 0.5 survived alevins or mobile *D. magna* or the EC_{50} values was estimated.

RESULTS

Field Water Analysis. At the sampling occasions during the peak snowmelt period, all water samples contained detectable levels of batatasin-III with the lowest values in the control stream (E) (Figure 2). The highest concentration (1.06 mg l^{-1}) was found in 1998 in snowmelt that drained from *E. hermaphroditum* vegetation adjacent to the lake at Akkanålke (H). At most locations with elevated levels, concentrations were lower at later sampling occasions, i.e., after the peak snowmelt period. In the water samples from locations with relatively high levels of batatasin-III, the pH was close to 4.5, but varied between pH 5.8 and 6.6 in the control stream. It was also evident that pH in all water samples was lowest during the peak snowmelt period and increased with time.

Survival of Brown Trout Alevins. There was a significant ($P < 0.001$) effect of batatasin-III concentration and exposure time ($P < 0.001$) on the proportion of live brown trout alevins (Table 1). Survival plots of the proportion of surviving alevins and batatasin-III concentration gave EC_{50} values of 10 mg l^{-1} after 24 hr and 2 mg l^{-1} after 48 hr, respectively (Figure 3). All alevins survived after 24-hr exposure to phenol at corresponding levels (data not shown), and the effect was negligible after 48-hr exposure (Figure 3). Oxygen levels in the experimental aquaria ranged between 82 and 76% O_2, and the pH remained stable within a ± 0.2 range.

Mobility of Dapnia magna. The frequency of mobile *D. magna* decreased significantly ($P < 0.001$) with increasing batatasin-III concentration, decreasing pH, and increasing exposure time (Table 2). The plotted functions of the proportion of mobile *D. magna* and concentration of batatasin-III at pH 5.5, 6.0, 6.5, and 7.0 gave EC_{50} values ranging between 7 and 17 mg l^{-1} after 24 hr and between 2.5 and 12 mg l^{-1} after 48 hr at pH 5.5 and pH 7.0, respectively (Figure 4). At pH 5.5,

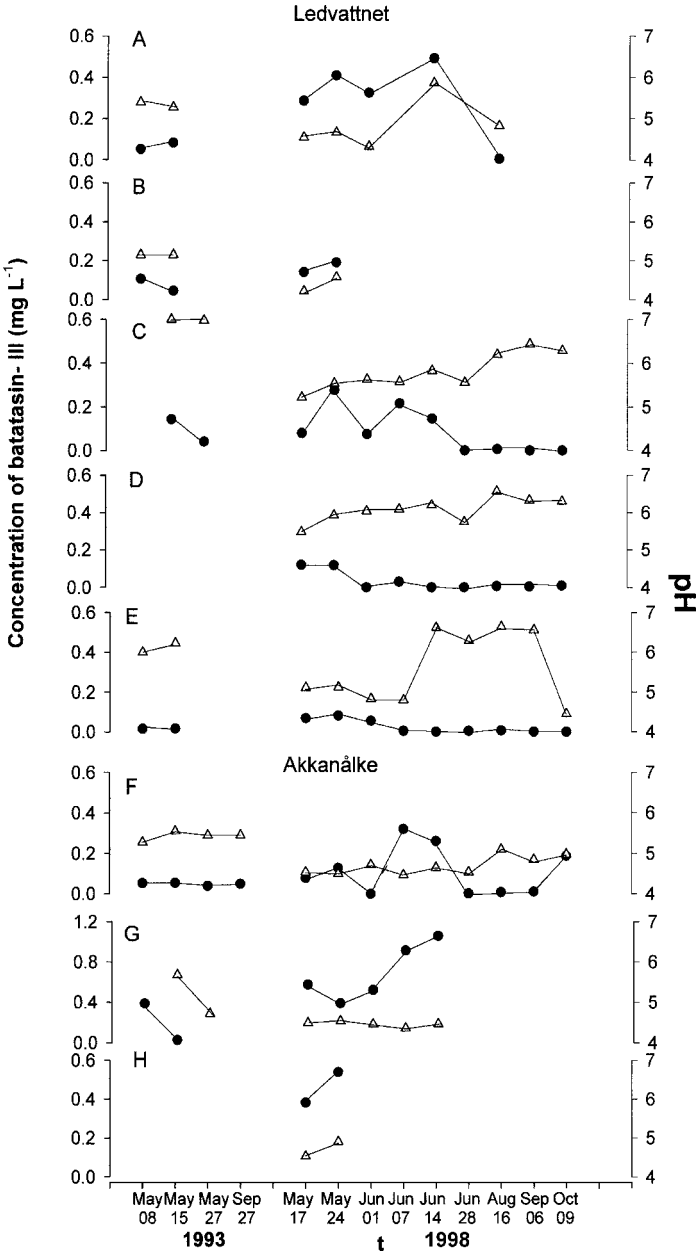

FIG. 2. Concentration of batatasin-III (mg l^{-1}) (filled circles) and pH (open triangles) in water samples collected at Levattnet forest drainage basin (sites A–E) and at Akkanålke Forest Mountain Reserve (sites F–H), N Sweden in 1993 and 1998. For a detailed description of sites, see Figure 1. Each plot is the mean of duplicate samples.

TABLE 1. ESTIMATED VALUES OF THE BIONOMIAL PROBIT MODEL OF BROWN TROUT

Variable	Coefficient	Standard error	*P*
Constant	7.341	2.07	
Concentration	−0.738	0.20	<0.001
Time	−5.968	1.72	<0.001

Note. Time = 1 for 48 hr and Time = 0 for 24 hr, the probit model gave $\chi^2 = 158.8$, df = 2, $P < 0.001$.

only 10–30% of the *D. magna* were mobile after 48 hr in the control treatment without batatasin-III, indicating a general, high sensitivity to low pH. The pH values remained stable (±0.1) throughout the 48-hr exposure time. The oxygen level was 84% saturation at all four pH levels from the start and ranged between 78 and 72% oxygen saturation at the end of the 48-hr exposure time.

DISCUSSION

This study shows that batatasin-III, the main secondary metabolite of *E. hermaphroditum*, is toxic to brown trout alevins and *D. magna* under controlled

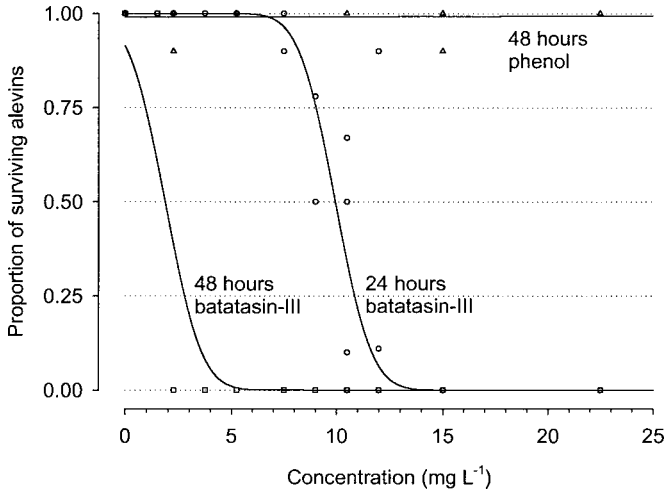

FIG. 3. Dose-response plots of the proportion of surviving newly hatched brown trout yolk sac alevins and the concentration of batatasin-III at pH 6.5 after 24 hr (open circles) and 48 hr (open squares). The plots are based on four replicates for each concentration. The corresponding proportion of surviving alevins after 48-hr exposure to phenol are indicated by open triangles.

TABLE 2. ESTIMATED VALUES OF THE BIONOMIAL PROBIT MODEL OF *Daphnia magna*

Variable	Coefficient	Standard error	*P*
Constant	−9.508	1.65	
Concentration	−0.318	0.04	<0.001
pH	2.110	0.32	<0.001
Time	−1.316	0.26	<0.001

Note. Time = 1 for 48 hr and Time = 0 for 24 hr, the probit model gave $\chi^2 = 180.5$, df = 3, $P < 0.001$.

conditions. The lethal effect of batatasin-III is both dependent on concentration and the time of exposure. No apparent effect was found when exposing brown trout alevins to phenol, which suggests a specific toxic component in the bibenzyl structure of batatasin-III (cf. Nilsson et al., 2000). The physiological action of batatasin-III on eucaryotic cells is unknown but its effect on fish appears similar to the well-known plant-produced toxic compound rotenone (which is also of phenolic origin). Rotenone kills fish by blocking the electron transfer at the mitocondrial level thereby inhibiting oxygen utilization (Ramos et al., 1996), and the fish suffocates (Davies and Shelton, 1983). The toxicity of rotenone is both concentration- and species-dependent (Bouck and Ball, 1965). However, phenol compounds like batatasin-III often are uncouplers of ATP formation (Ramos et al., 1996). Fish have a higher sensitivity to rotenone compared to crustaceans (Davies and Shelton, 1983). If the mode of actions of the two compounds is comparable, this may explain why batatasin-III had a lower EC_{50} value on fish than on *D. magna* in this study.

The interactive affect of pH on the toxicity of batatasin-III was pronounced in the experiments with *D. magna*. Toxic effects of phenols are clearly amplified by acidification (Horner et al., 1988). Snowmelt during spring in northern boreal areas results in a substantially lowered pH of the water in streams, so-called "acid shocks," and this coincides with the peak levels of batatasin-III found in water samples in this study. Unfortunately, exposing brown trout alevins to batatasin-III at different pH levels, in particular as low as 4.5, was beyond the budget of this study. Cultured *D. magna* is also sensitive to pH lower than 5.5, and, therefore, we were unable to test the toxicity of batatasin-III under truly realistic pH conditions. However, since the biological activity of phenolics is, in general, highest at pH 3–4 (Horner et al., 1988), and pH as low as 4.0 is commonly found in nature, it is likely that we have underestimated the negative effects of batatasin-III during snowmelt period.

Batatasin-III was first identified as a dormancy-inducing compound in yam (*Discorea batatas*) bulbils (Hashimoto et al., 1972), and was later isolated from a number of different genera of *Orchidaceae* (Veerraju et al., 1989; Majumder and Basak, 1991; Majumder and Pal, 1992; Majumder and Ghosal, 1993; Bai et al.,

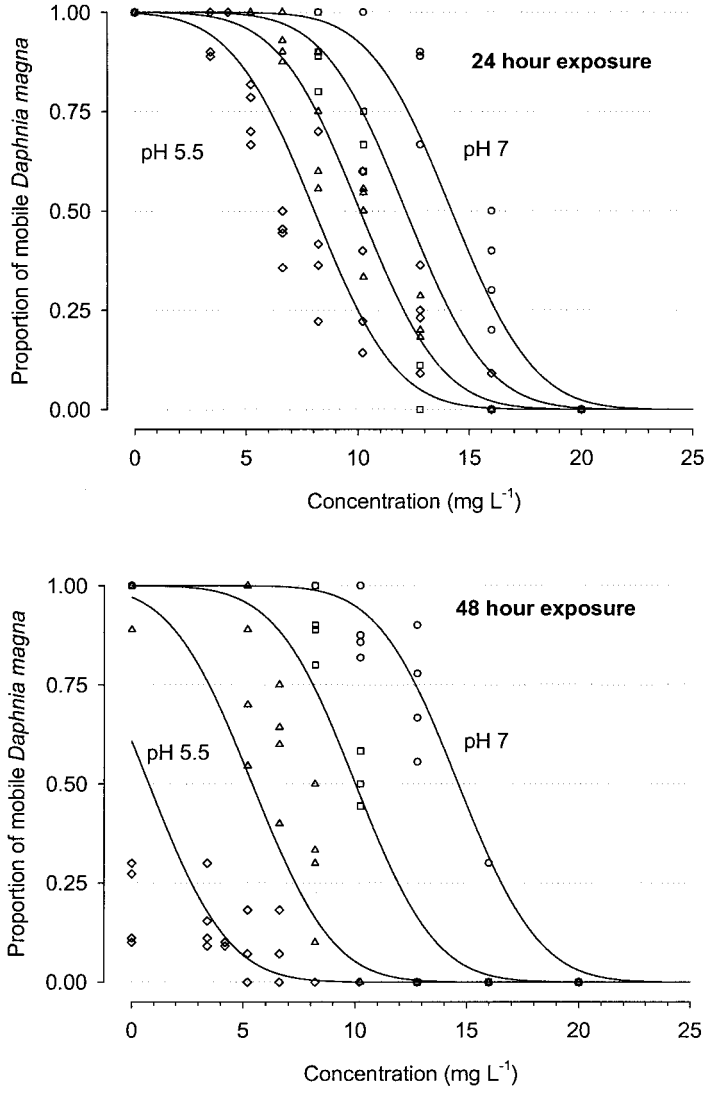

FIG. 4. Dose-response plots on the acute effects of batatasin-III on juvenile *Daphnia magna* after 24 hr (above) and 48 hr (below) exposure. The plots are based on four replicates for each concentration of batatasin-III. The acute toxicity tests were done at pH 7.0 (open circles), pH 6.5 (open squares), pH 6.0 (open triangles), and pH 5.5 (open diamonds), respectively.

1997). To our knowledge, batatasin-III has thus far only been found in one plant species (*E. hermaphroditum*) in the boreal forest. The levels of batatasin-III found in streams, runnels, and the lake in this study are, therefore, likely to originate from terrestrial surroundings. The maximum levels of batatasin-III detected in the field were lower than the EC_{50} values estimated for both trout and *D. magna*, but were in the lower range of those causing lethal effects on fish and immobilization of *D. magna*. This was particularly true at low pH in the acute toxicity tests. However, it is extremely difficult with single sampling occasions to determine the actual concentration that the response organisms will be exposed to. The duration of high levels of batatasin-III appears to be several weeks in streams and lakes. Also, batatasin-III is fat-soluble, and lipophilic compounds may accumulate in membranes to give concentrations far exceeding the concentration in the external solution (cf. Escher et al., 1996; Wallstedt et al., 2001). At present, there are no available data on the level of the compounds in the tissue of the fish after long exposure. It is possible that the total amount of available batatasin-III in streams and lakes (and not the concentration) is critical for determining the inhibiting potential caused by this compound.

Statistics from the Swedish National Forest Survey indicate that *E. hermaphroditum* is the most abundant plant species on clear-cuts in the province of Lapland of northern Sweden. It is naturally fire sensitive and has been favored by forest fire suppression during the past 100 years (Zackrisson, 1977), and by selective cutting prior to the 1950s especially in nutrient poor sites at altitudes above 250 m (Nilsson, 1992). Forest fire is the only known method to reverse the negative effects of combusting plant and humic material, increasing pH, and producing charcoal that functions as a natural adsorbent agent for batatasin-III and other phenolic compounds (Zackrisson et al., 1996; Wardle et al., 1998; DeLuca et al., 2002).

This study highlights the potential ecological causes of a shift from fire disturbed to anthropogenic disturbed forest ecosystems. Forest companies and governmental organizations have invested and intend to invest large resources into restoring disturbed aquatic ecosystems to improve recreational fishing. There is an increasing awareness of the importance of small streams as nursery areas for fish and as contributors to biological diversity in general. Data from this study imply that batatasin-III leached from the drainage areas of small streams may cause mortality of brown trout alevins and that major food (such as *D. magna*) of fish is also sensitive to exposure of batatasin-III. However, these findings have to be kept in perspective until further studies have been performed. Extensive field sampling is needed before we can evaluate whether the increasing abundance of *E. hermaphroditum* will release concentrations of batatasin-III that are high enough to be a threat to aquatic organisms. Future experimental studies have to focus upon the combined effects of low pH, iron, and humic acid together with observed concentrations of batatasin-III and the effects of long time exposure as well. The metabolite, batatasin-III also has a structural resemblance to steroids. In view of

the increasing focus on endocrine disruption in wildlife, such as estrogenic effects of sewage on fish (Taylor and Harrison, 1999), a functional resemblance of batatasin-III to estrogen should be studied as well.

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ACQUIRED AND PARTIALLY *DE NOVO* SYNTHESIZED PYRROLIZIDINE ALKALOIDS IN TWO POLYPHAGOUS ARCTIIDS AND THE ALKALOID PROFILES OF THEIR LARVAL FOOD-PLANTS

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Abstract—The profiles of pyrrolizidine alkaloids (PAs) in the two highly polyphagous arctiids *Estigmene acrea* and *Grammia geneura* and their potential PA sources in southeastern Arizona were compiled. One of four species of Boraginaceae, *Plagiobothrys arizonicus*, contained PAs; this is the first PA record for this plant species. The principle PA sources are *Senecio longilobus* (Asteraceae) and *Crotalaria pumila* (Fabaceae). The known PA pattern of *S. longilobus* was extended; the species was found to contain six closely related PAs of the senecionine type. Three novel PAs of the monocrotaline type, named pumilines A–C, were isolated and characterized from *C. pumila*, a species not studied before. The pumilines are the major PAs in the seeds, while in the vegetative organs they are accompanied by the simple necine derivatives supinidine and as the dominant compound subulacine (1β,2β-epoxytrachelanthamidine). In both plant species, the PAs are stored as *N*-oxides, except *C. pumila* seeds, which accumulate the free bases. Great variation in PA composition was observed between local populations of *C. pumila*. The PA profiles were established for larvae and adults of *E. acrea* that as larvae had fed on an artificial diet supplemented with crotalaria-powder and of *G. geneura* fed with *S. longilobus*. In both experiments, the larvae had a free choice between the respective PA source and

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diet or food plants free of PAs. The profiles compiled for the two species reflect the alkaloid profiles of their PA sources with one exception, subulacine could never be detected in *E. acrea*. Besides acquired PAs, insect PAs synthesized from acquired necine bases and necic acids of insect origin were detected in the two arctiid species. These insect PAs that do not occur in the larval food sources accounted for some 40–70% (*E. acrea*) and 17–37% (*G. geneura*) of total PAs extracted from the insects. A number of novel insect PAs were identified. Plant-acquired and insect PAs were found to accumulate as *N*-oxides. The results are discussed in relation to specific biochemical, electrophysiological, and behavioral mechanisms involved in PA sequestration by arctiids.

Key Words—Arctiidae, *Crotalaria, Senecio,* pyrrolizidine alkaloid, alkaloid sequestration, insect alkaloid, chemical defense.

INTRODUCTION

Pyrrolizidine alkaloids (PAs) are a class of secondary metabolites, produced by plants that are known to be toxic and/or deterrent to various animals. Insect herbivores that are specialized to feed on such plants often sequester the PAs and store them in their bodies. In several cases, the acquired PAs are known to provide protection from certain predators. In addition, some species utilize the acquired PAs as precursors for the formation of pheromones and insect-specific retronecine esters (for review see Hartmann and Witte, 1995; Schulz, 1998; Hartmann and Ober, 2000).

The ability to sequester PAs appears to be phylogenetically widespread. Certain chrysomeline leaf beetles sequester them as larvae and adults (Ehmke et al., 1999; Pasteels et al., 2003). Among Lepidoptera, danaine and ithomiine butterflies obtain PAs pharmacophagously from dead or decaying organs of alkaloid-containing plant species (Boppré, 1990). Many arctiid moths sequester PAs as larvae from PA-containing host plants (Weller et al., 1999), while others, especially in the subfamily Ctenuchinae, collect them as adults. In any case, species obtaining PAs from plants prevent accumulation of the toxic PA free base (Lindigkeit et al., 1997; Hartmann et al., 1999, 2001). Most PA-adapted species store the alkaloids as nontoxic *N*-oxides.

Most PA-sequestering arctiid larvae studied in detail are monophagous or narrowly oligophagous species. They have specialized diets of PA-containing plants, which they utilize during their larval life span as both nutrient and PA source. Examples are *Tyria jacobaeae* feeding on *Senecio jacobaea* (Aplin and Rothschild, 1972; Rothschild et al., 1979; Ehmke et al., 1990; Nickisch-Rosenegk and Wink, 1993) and *Utetheisa ornatrix* feeding on *Crotalaria* spp. (Conner et al., 1990; Dussourd et al., 1991). These species conform to the expected pattern of host-specialized insect herbivores that have acquired particular abilities to make use of plant compounds specific to their hosts.

By contrast, there are species of arctiids that are extreme generalists and include PA-containing plants in their diets. Among these are *Estigmene acrea* and *Grammia geneura.* For *E. acrea,* 69 host plant species are listed (Robinson et al., 2002) of which two are known to contain PAs. For *G. geneura,* 82 food plant species have been recorded during field observations in southeastern Arizona (Singer, 2000), including one *Crotalaria* species, one *Senecio* species, and species from four genera of the Boraginaceae, all of which are expected to contain PAs. For the caterpillars of both *E. acrea* and *G. geneura,* PA-containing species are not reliably abundant in the habitat and may be encountered only rarely. However, the PAs are strong phagostimulants for caterpillars of both species, and both of them have highly specialized taste receptors that can detect low PA concentrations (Bernays et al., 2002a,b), suggesting that the PAs are of singular importance in their biology.

Larvae of *E. acrea* are known to sequester PAs and transfer them into the adult stage, and males were shown to synthesize from PAs the short-distance mating pheromones hydroxdanaidal and danaidal that are emitted from the coremata (Krasnoff and Roelofs, 1989). Our preliminary studies indicate that *G. geneura* larvae also sequester PAs and transfer them to the adult stage, but nothing is known about PA-derived pheromones. However, they have no coremata and it is assumed that they do not use PAs in reproductive behavior (Ferguson, 1985).

In this paper, we (i) identified and characterized the alkaloids found in the PA-containing plant species growing in the natural habitat of *E. acrea* and *G. geneura* in southeastern Arizona; (ii) characterized the profiles of PAs acquired from plants, in larvae and adults of *E. acrea* and *G. geneura*; (iii) identified and characterized a number of known and novel alkaloids synthesized by the arctiids from plant-acquired necine bases and necic acids of their own (insect PAs). The results indicate that in these two extreme generalists, gustatory sensitivity to PAs is matched by specialized biochemistry of PA sequestration and metabolism, indicating specific adaptations for utilization of these compounds.

METHODS AND MATERIALS

Insects. Caterpillars and adults of *Estigmene acrea* (Drury) were obtained from cultures reared in the laboratory on artificial diet with PAs present. Larvae were given the plain wheat-germ-based diet (Yamamoto, 1969) and, in addition, the same diet mixed with dry *Crotalaria pumila* powder at 10% of the dry weight. This powder contained PAs at a level of 0.18%, giving a final concentration in the diet of 0.018%. Individuals could eat from both foods *ad libitum,* but ate more of the PA-containing diet. After the PA treatment, larvae to be analyzed were confined with plain diet for 24 hr to ensure that PAs in the gut were evacuated, and all measured PAs were in tissues. Other larvae were allowed to complete development with the

choice of diet, and the adults that finally emerged were analyzed for PAs. Both larvae and adults were killed by freezing and stored at −20°C.

Caterpillars (penultimate or final instars) of *Grammia geneura* (Strecker) were collected from a field population where *S. longilobus* Benth. and *Ambrosia confertiflora* (DC.) Rydb. were the only abundant host-plants. The larvae were further reared on a host-plant mixture of *Malva parviflora* L., *S. longilobus,* and *A. confertiflora.* One cohort of larvae in the final instar was transferred to plain synthetic diet for 24 hr to evacuate the gut of PAs and then frozen. Others were allowed to pupate, and adults were frozen within 24 hr of emergence.

Insect samples allotted to PA analysis were freeze-dried and kept in closed vials until analysis.

Plants. All plants were collected at field sites in southeastern Arizona as indicated. Plants were either air-dried at temperatures of 20–40°C and R.H. <20%, except in the case of *C. pumila* collected from Montosa Canyon, which was freeze-dried and preserved until analysis. Plants selected for analysis were those observed to be most acceptable to larvae in nature as a result of approximately 300 hr of continuous observations in the field (Singer, 2000; unpublished observations).

Sample Preparation for GC and GC-MS Analysis. (Witte et al., 1993). Dried plant tissue was ground, and 400 mg were extracted in 5 ml 1 M HCl for 2–3 hr and centrifuged; the pellet was dissolved in 1 ml 1 M HCl and again extracted. The combined supernatants were extracted with 2 ml dichloromethane, and the aqueous phase was recovered; half of the solution was made basic with 25% ammonia and applied to an Extrelut (Merck) column-3 (1.4-ml solution/g Extrelut). The alkaloid free bases were eluted with dichloromethane (6 ml/g Extrelut). The solvent was evaporated, and the residue dissolved in 10–100 μl methanol prior to GC or GC-MS. The remaining half of the acid supernatant was mixed with an excess of Zn dust and stirred for 3 hr at room temperature for complete reduction of alkaloid *N*-oxides. Then, the mixture was made basic and further processed as above. This fraction contains total PAs (PA free bases and PA *N*-oxides).

Single freeze-dried insects were weighed and then ground in 0.2–2 ml 1 M HCl in a mortar, and further processed as described for plant samples. The size of the Extrelut columns (1.0–3.0 ml) was adapted to the sample volume of the extracts.

Routine Gas Chromatography. Separation was achieved using a capillary column (15 m × 0.25 mm fused-silica; DB-1, J&W Scientific) (Witte et al., 1993). All other GC conditions were the same as given for GC-MS. Detectors were FID and PND. Quantitative analyses were performed via the FID signals with heliotrine as internal standard.

Isolation of Pyrrolizidine Alkaloids from Crotalaria pumila. For the isolation and separation of the pumilines A–C, 50 g powdered seeds, obtained in October 2002 from a *C. pumila* population growing at Arivaca Creek Buenos Aires wildlife Refuge (Arizona), were extracted with petroleum ether in a Soxhlet apparatus for

24 hr. This method allowed the separation of the more polar PAs, i.e., supinidine and subulacine, which are not extracted into petrolium ether. The solvent was removed, the crude extract suspended in 100 ml 1 M HCl and washed twice with dichloromethane, made alkaline with ammonia, applied to an Extrelut (Merck) column, and the alkaloid fraction was eluted with dichloromethane. The alkaloid fraction (124 mg) was applied to a 16 × 1.5 cm column filled with silica gel 30 (Merck 9385). Elution with the solvent dichloromethane/methanol (95:5 by vol.) allowed the separation of pumiline A (fraction I) and pumiline B (fraction II); both fractions, however, were still contaminated with pumiline C and PA-X1 (M^+ 335). Complete separation was achieved by using preparative HPLC. Samples of 0.5 ml were applied to a RP-18 column (Nucleosil 120-7, 250-mm long, 25 mm i.d.; Macherey & Nagel). Separation was achieved isocratically by using acetonitrile/trifluoroacetic acid (25:75 by vol.), pH 2, at a flow rate of 9 ml · min^{-1}; detection was by absorbance at 205 nm. Separation of fractions I and II provided three compounds each, fraction I: pumiline A (t_R13.8 min), PA-X1 (t_R16.9 min), and pumiline C (t_R20.1 min), fraction II: pumiline B (t_R14.3 min), PA-X1 (t_R16.9 min), and pumiline C (t_R20.1 min). Recovery: pumiline A, 12 mg; pumiline B, 15 mg; and pumiline C, 10 mg; PA-X1 could not be recovered in sufficient amounts for NMR analysis.

For the isolation of subulacines, 8.4 g dried leaves were extracted in 2 M HCl. The aqueous fraction was washed twice with diethyl ether. The remaining aqueous phase was reduced with an excess of zinc dust for 3 hr, made alkaline with ammonia, and purified via Extrelute as decribed above. Pure subulacine (5.2 mg) was obtained by column chromatography with silica gel 60 (Merck).

NMR Spectroscopy. NMR spectra were obtained on a Bruker Avance 400 spectrometer at operation frequencies of 400 MHz (^{1}H) and 101 MHz (^{13}C), respectively. The solvent was $CDCl_3$, and the chemical shifts were referenced to TMS ($\delta_H = 0.00$) and to $CDCl_3$ ($\delta_C = 77.0$). The spectrometer software used was XWinNMR version 3.1. The manufacturer's pulse programs "invigstp" (gradient-selected phase-sensitive HSQC), "inv4gslplrdn" (gradient-selected HMBC), "cosygp" (gradient-selected COSY), and "noesyrvtp" (phase-sensitive NOESY using TPPI) controlled the two-dimensional NMR experiments. The H,C-correlation experiments were optimized for coupling constants of 145 Hz [^1J(C,H)] and 7.7 Hz [$^{2,3}J$(C,H)]. Digital resolutions in both dimensions were chosen small enough to permit the resolution of closely lying chemical shifts. The mixing time in the NOESY experiments was 2.0 sec.

Gas Chromatography-Mass Spectrometry (GC-MS). The GC-MS data were obtained with an Hewlett Packard 5890A gas chromatograph equipped with a 2 m fused silica guard column (deactivated, I.D. 0.32 mm) and a 30 m × 0.32 mm analytical column (ZB1 and ZB5, Phenomenex). The capillary column was directly coupled to a triple quadrupole mass spectrometer (TSQ 700, Finnigan). The conditions applied were as follows: Injector and transfer line were set at 280°C;

the temperature program used was 100°C (3 min) – 300°C at 6°C · min^{-1} for PA separation and 70°C (6 min) – 300°C at 10°C · min^{-1} for the separation of necic acid methyl esters, respectively. The injection volume was 1 μl. Depending on concentration, the split ratio was 1:20 or splitless, the carrier gas flow was 1.6 ml · min^{-1} He, and the mass spectra were recorded at 70 eV. CI mass spectra were recorded in the positive mode with the same GC-MS system using ammonia as a reagent gas. The identification of the necic acids of the insect PAs was achieved by transesterification with trimethylsulfoniumhydroxide (TMSH, Fluka, Seelze, Germany) by the following standard procedure: crude methanolic PA extracts were dried under nitrogen, resuspended in 10 μl tertiary-butyl-methylether, and 10 μl 0.2 M TMSH were added. After 10 min at room temperature, the mixture was directly applied to GC-MS. Mass spectra of the methylated necic acids were compared to data obtained from authentic aliphatic 2-hydroxy acids (Aldrich, Seelze, Germany) analyzed under identical conditions.

The retention index (RI) was calculated by a set of hydrocarbons (even numbered C_{12} to C_{28}) by linear interpolation. RIs obtained by GC-MS were proportionally higher than those obtained by routine gas chromatography caused by the different column length and origin. Therefore, the column identity is always indicated when RI values are given.

GC-MS Data of PAs from C. pumila. *Pumiline B:* RI(ZB1) 2172, RI(ZB5) 2225 GC-EIMS, m/z (rel. int.): 323 (3, $[M^+]$), 308 (0.5), 236 (8), 222 (10), 138 (27), 137 (23), 136 (73), 121 (37), 120 (78), 119 (100), 118 (10), 108 (11), 106 (11), 95 (48), 94 (31), 93 (47), 83 (8), 80 (17), 53 (7), 43 (22), 41 (7). GC-CIMS (NH_3): 326 (3), 325 (18), 324 (100, $[M+H]^+$), 280 (2), 120 (4).

Pumiline C: RI(ZB1) 2266, RI(ZB5) 2315GC-EIMS, m/z (rel. int.): 337 (2, $[M^+]$), 308 (1.5), 222 (11), 138 (26), 137 (29), 136 (72), 122 (6), 121 (41), 120 (78), 119 (100), 118 (10), 109 (7), 108 (10), 106 (10), 95 (42), 94 (28), 93 (41), 83 (9), 80 (15), 69 (6), 67 (6), 57 (14), 55 (5). GC-CIMS (NH_3): 339 (20), 338 (100, $[M+H]^+$), 294 (3), 120 (6).

Pumiline A: RI(ZB1) 2370, RI(ZB5) 2430 GC-EIMS, m/z (rel. int.): 351 (3, $[M^+]$), 264 (7), 236 (7), 140 (6), 139 (5), 138 (19), 137 (17), 136 (72), 123 (5), 122 (23), 121 (25), 120 (100), 119 (93), 118 (12), 109 (6), 108 (12), 106 (10), 95 (23), 94 (37), 93 (45), 81 (5), 80 (18), 67 (7). GC-CIMS (NH_3): 353 (19), 352 (100, $[M+H]^+$), 336 (6), 120 (8).

Subulacine (1β,2β-epoxy-trachelanthamidine): RI(ZB1) 1282, RI(ZB5) 1318. GC-EIMS, m/z (rel. int.): 155 (42, $[M^+]$), 126 (6), 124 (20), 96 (9), 71 (4), 70 (100), 68 (4), 55 (27), 42 (8), 41 (10).

GC-MS Data of the Insect PAs. Estigmine A {9-(2-hydroxy-3-methylbutanoyl)-supinidine}: RI(ZB1) 1736, RI(ZB5) 1782. GC-EIMS, m/z (rel. int.): 239 (0.5, $[M^+]$), 123 (9), 122 (100), 121 (39), 120 (50), 108 (17), 94 (6), 93 (31), 80 (14), 55 (7), 53 (9), 43 (5), 41 (7). GC-CIMS (NH_3): 241 (13), 240 (100, $[M+H]^+$), 124 (33), 122 (14), 140 (10).

Estigmine B {*9-(2-hydroxy-3-methylpentanoyl)-supinidine*}*:* RI(ZB1) 1835, *RI*(ZB5) 1885. GC-EIMS, *m*/*z* (rel. int.): 253 (0.5, [M^+]), 123 (10), 122 (100), 121 (44), 120 (52), 108 (17), 94 (6), 93 (26), 80 (12). GC-CIMS (NH_3): 255 (15), 254 (100, [M+H]$^+$), 124 (10), 122 (6).

Isocreatonotine A {*7-(2-hydroxy-3-methylbutanoyl)-retronecine*}*:* RI(ZB1) 1864, RI(ZB5) 1902. GC-EIMS, *m*/*z* (rel. int.): 255 (4, [M^+]), 237 (29), 138 (41), 137 (25), 136 (16), 124 (23), 120 (29), 111 (84), 106 (52), 94 (20), 80 (100).

Creatonotine A {*9-(2-hydroxy-3-methylbutanoyl)-retronecine*}*:* RI(ZB1) 1886, RI(ZB5) 1928. GC-EIMS, *m*/*z* (rel. int.): 255 (3, [M^+]), 211 (7), 139 (9), 138 (100), 137 (13), 136 (8), 94 (26), 93 (63), 80 (6), 55 (3).

Isocreatonotine B {*7-(2-hydroxy-3-methylpentanoyl)-retronecine*}*:* RI(ZB1) 1962, RI(ZB5) 2005. GC-EIMS, *m*/*z* (rel. int.): 269 (6, [M^+]), 251 (26), 138 (68), 137 (30), 136 (17), 124 (20), 120 (29), 111 (75), 106 (56), 94 (30), 80 (100).

Creatonotine B {*9-(2-hydroxy-3-methylpentanoyl)-retronecine*}*:* RI(ZB1) 1985, RI(ZB5) 2030. GC-EIMS, *m*/*z* (rel. int.): 269 (2, [M^+]), 225 (6), 139 (10), 138 (100), 137 (14), 136 (8), 120 (3), 95 (4), 94 (23), 93 (56), 80 (6).

Callimorphine {*9-(2-acetoxy-2-methylbutanoyl)-retronecine*}*:* RI(ZB1) 1964, RI(ZB5) 2012. GC-EIMS, *m*/*z* (rel. int.): 297 (1, [M^+]), 155 (11), 154 (17), 138 (65), 137 (20), 136 (14), 94 (28), 93 (100), 80 (11), 73 (8), 43 (17).

Homocallimorphine {*9-(2-O-propionyl-2-methylbutanoyl)-retronecine*}*:* RI (ZB1) 2044, RI(ZB5) 2099. GC-EIMS, *m*/*z* (rel. int.): 311 (1, [M^+]), 155 (12), 154 (21), 139 (7), 138 (70), 137 (24), 136 (17), 94 (27), 93 (100), 80(9), 57 (17).

Deacetylcallimorphine {*9-(2-hydroxy-2-methylbutanoyl)-retronecine*}*:* RI (ZB1) 1830. GC-EIMS, *m*/*z* (rel. int.): 255 (0.5, [M^+]), 139 (13), 138 (100), 137 (15), 136 (8), 95 (6), 94 (35), 93 (78), 80 (11), 73 (13), 44 (8).

RESULTS

Pyrrolizidine Alkaloids (PAs) of the Larval Food Plants. Among the plants that are favored food plants of *Estigmene acrea* and *Grammia geneura* in southern Arizona, only three contained PAs. In *Plagiobothrys arizonicus* (A. Gray) Greene ex A. Gray (Boraginaceae), a species not yet known to contain PAs, small quantities (total 0.007% of the dry weight) of alkaloids of the lycopsamine type (Hartmann and Witte, 1995) were identified by GC-MS (Table 1). No PAs could be detected in leaves of a second Boraginaceae species, *Cryptantha barbigera* (A.Gray) Greene, belonging to a genus with many PA-containing species (Stermitz et al., 1993).

Senecio longilobus Benth. (syn. *S. douglasii* DC. var. *longilobus* (Benth.) L. Benson) is a well-known poisonous plant in the southwestern United States. GC-MS analysis of flower heads and leaves revealed six PAs (Table 2) that could be unequivocally identified according to their RI values, molecular mass, and mass fragmentation patterns in comparison to reference compounds. The occurrence

TABLE 1. PYRROLIZIDINE ALKALOIDS OF *Plagiobothrys arizonicus*, IDENTIFIED BY GC-MS

Alkaloid	RI (DB-1)	$[M]^+$ m/z	Rel. abundance (%)
Indicine	2119	299	tr
Lycopsamine	2132	299	49
7-Acetyl-lycopsamine	2220	341	35
3'-Acetyl-lycopsamine	2242	341	8
3',7-Diacetyl-lycopsamine	2332	383	8
Total PAs (mg · g^{-1} dry weight): 0.074			

Note. RI: retention index.

of senecionine, integerrimine, seneciphylline, and retrorsine in *S. longilobus* was known (Ray et al., 1987; Cooper et al., 1996). In addition, spartioidine and usaramine, the diasteromers of seneciphylline and retrorsine were identified as new PAs for *S. longilobus*. These six alkaloids (Figure 1) are among the most frequent PAs in *Senecio* spp. (Hartmann and Witte, 1995). All alkaloids are present in the plant as *N*-oxides. The concentrations are high reaching over 1% of the dry weight in leaf samples for one site.

Crotalaria pumila is an annual herb. The only report on alkaloids in this species refers to trace amounts of monocrotaline (Williams and Molyneux, 1987). GC-MS analysis revealed the absence of monocrotaline but instead the presence of at least four unknown PAs of the monocrotaline type, as well as two simple necine derivatives, supinidine and the 1,2-epoxide subulacine (Figure 1). Supinidine was identified by its RI, molecular mass, and fragmentation pattern in comparison to a reference compound, and subulacine (1β,2β-epoxytrachelanthamidine)

TABLE 2. PYRROLIZIDINE ALKALOIDS IN TWO POPULATIONS OF *Senecio longilobus* FROM SOUTHEASTERN ARIZONA

			Relative abundance (%)		
			Patagonia mountains		Box canyon
Alkaloid	RI (ZB-1)	$[M]^+$	Flower heads	Leaves	Leaves
Senecionine	2294	335	2	1	2
Seneciphylline	2310	333	10	13	18
Spartioidine	2346	333	14	16	15
Integerrimine	2353	335	15	25	14
Retrorsine	2522	351	16	24	23
Usaramine	2584	351	38	17	22
Total PA (mg · g^{-1} dry weight):			4.1	11.2	3.9

Note. RI: retention index.

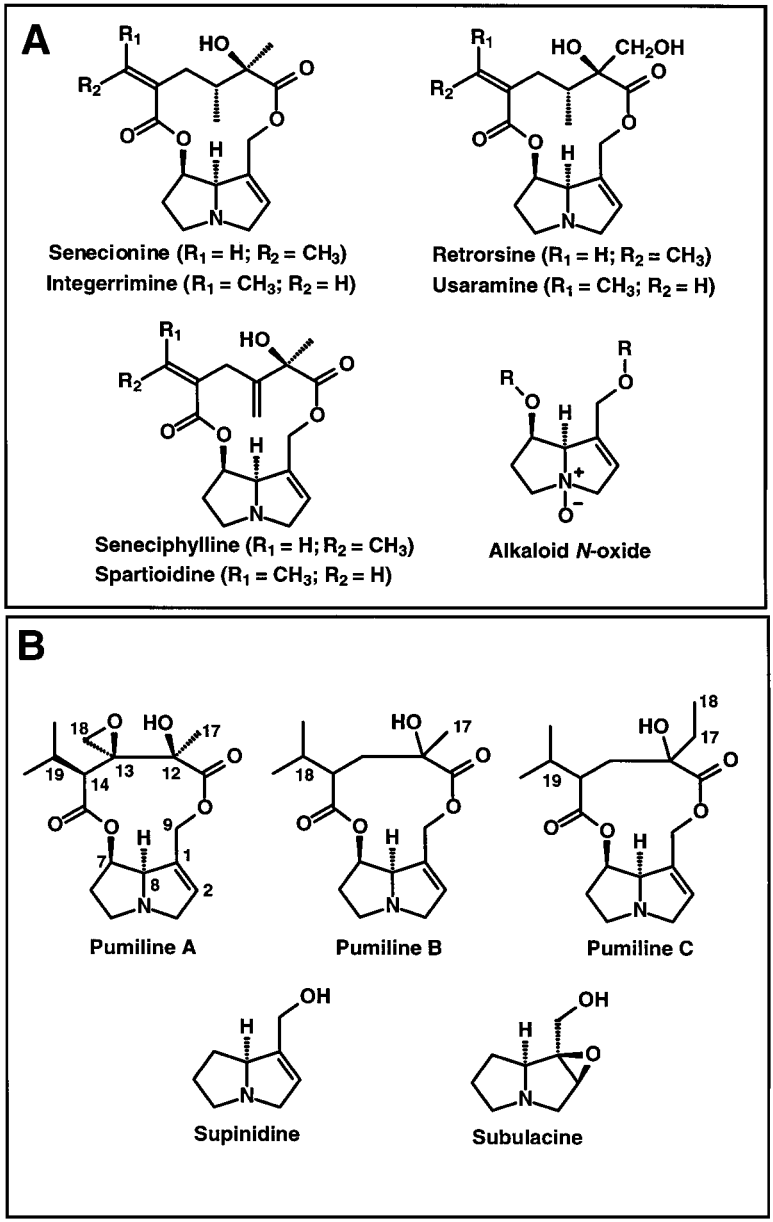

FIG. 1. Structural formulae of the pyrrolizidine alkaloids (PAs) identified from (A) *Senecio longilobus* and (B) *Crotalaria pumila*. In the plant, the PAs are present as *N*-oxides, except in the *Crotalaria* seeds. Relative configurations in the macrocyclic ring of pumiline A are tentative.

was identified by its ^{1}H and ^{13}C NMR data that were identical with those published previously. The alkaloid was first characterized from *Crotalaria trifoliastrum* (Culvenor et al., 1967), and was also found in the Boraginaceae *Heliotropium subulatum* (Stermitz and L'Empereur, 1988). Three of the unknown macrocyclic alkaloids detected by GC-MS were isolated and analyzed by ^{1}H and ^{13}C NMR analysis. They are novel alkaloids of the monocrotaline type and were named pumiline A, B, and C. Basic hydrolysis of the pumilines revealed a single necine base that in GC-MS showed properties identical to authentic retronecine. Structure elucidation by NMR spectroscopy of the pumilines was achieved as follows.

Identification of Pumiline A (Table 3, Figure 1): The ^{1}H and ^{13}C NMR spectra of pumiline A were fully assigned by the use of the following techniques: ^{13}C-DEPT, $^{1}H,^{1}H$-COSY, $^{1}H,^{13}C$-HSQC, and $^{1}H,^{13}C$-HMBC. This allowed the constitution of the molecule to be derived unambiguously (Table 3). Characteristic features of the spiroepoxide group are the δ_C value of 48.2 for the CH_2-carbon together with geminal J(H,H) and one-bond J(C,H) coupling constants of 4.3 and ca. 179 Hz, respectively. The latter was obtained from the ^{13}C satellites in the ^{1}H spectrum. The isopropyl group at C-14 is recognized from its diastereotopic methyl groups ($\delta = 1.05$ and 0.82) coupled to a common proton at $\delta = 1.66$ with a doublet of septets multiplicity ($J = 10.7$ and 6.5 Hz). An attempt was made to derive the configurations of the chiral centers by 2D H,H-NOESY. As no NOEs were observed between the protons of the necic acid and pyrrolizidine moieties, the configurations of the chiral centers of the acid could not be related to those of the base. Hence, C-12 was arbitrarily assumed to possess the usual (*R*)-configuration of monocrotalines. The NOESY crosspeaks relevant to the configurations of C-13 and C-14 relative to C-12 are those between one of the epoxide protons and H-14 and between the other epoxide proton and the protons of the methyl group connected to C-12. If interpreted in a straightforward manner, these findings suggest the relative configuration shown in the formula with C-17, C-18, and H-14 on the "underside" of the molecule. Dreiding molecular models, however, show that the macrocyclic ring of the pumilines is flexible. Hence, one cannot conclude with certainty that a molecule with a different configuration of C-14 and/or C-18 might assume a conformation that would also be compatible with the observed NOEs.

Identification of Pumiline B (Table 3, Figure 1): The NMR spectra of pumiline B show the absence of the spiroepoxide group at C-13. Instead, this carbon atom carries two hydrogens that are spin-coupled to H-14. The latter hydrogen is coupled to the methine proton of the isopropyl group. A NOESY crosspeak is observed between the protons of methyl group 17 and the more deshielded one (H-13a) of the protons at C-13. This proton and its geminal partner, H-13b, have vicinal coupling constants with H-14 of 8.2 and 1.1 Hz, respectively. Again, in view of the flexibility of the 11-membered ring, the mentioned NOE and coupling constants do not suffice to define unambiguously the relative configurations of C-12 and C-14.

TABLE 3. NMR DATA OF PUMILINES A–C

Position	δ_C	δ_H	HMBC crosspeaks
Pumiline A			
1	129.7		9a, 9b, (3a, 3b)
2	133.3	6.27[a]	9a, 9b, (3a)
3	59.2	4.52 (a), 3.81 (b)	5b
5	53.9	4.02 (a), 2.99 (b)	7, (6a)
6	34.2	2.46 (a), 2.15 (b)	5b
7	71.4	5.63	5a, 6b
8	77.7	5.21	5a, 6b, 7, 9a, (2, 9b)
9	60.7	5.26 (a), 4.27 (b)	
11	175.3		9a, 9b, 17
12	77.1		14, 17, 18a, 18b
13	59.8		14, 17, 18a, 18b
14	50.9	2.57	19, 20, 21
15	173.1		7, 14, (20)
17	21.7	1.25	
18	48.2	3.25 (a), 2.80 (b)	14, 17
19	27.5	1.66	14, 20, 21
20	21.9	1.05	14, 21
21	20.6	0.82	20
OH		ca. 3.2	
Pumiline B			
1	131.3		3b, 9a, 9b
2	135.7	6.18[a]	3b, 9a, 9b
3	60.9	4.09 (a), 3.58 (b)	2, 5b
5	54.2	3.50 (a), 2.75 (b)	3b, 7, (6a)
6	34.3	2.20 (a), 1.98 (b)	5b
7	71.9	5.64	6b
8	78.1	4.60	2, 6a, 7, 9a, 9b
9	60.8	5.15 (a), 4.25 (b)	2
11	176.5		9a, 9b, 13b, 17
12	74.5		13b, 14, 17
13	40.2	2.23 (a), 1.84 (b)	14, 17
14	47.8	2.03	13b, 19, 20, (6a, 17, 18)
15	176.1		7, 13a, 13b, 14
17	28.7	1.40	13b
18	31.6	1.78	13b, 14, 19, 20
19	20.03	0.89	14, 18, 20
20	19.97	0.96	14, 18, 19
OH		3.15	
Pumiline C			
1	130.9		9a, 9b
2	134.9	6.19[a]	9a, 9b
3	60.5	4.16 (a), 3.60 (b)	
5	54.1	3.59 (a), 2.76 (b)	7
6	34.2	2.24 (a), 2.00 (b)	

TABLE 3. CONTINUED

Position	δ_C	δ_H	HMBC crosspeaks
7	71.8	5.64	
8	78.0	4.69	9a
9	60.8	5.15 (a), 4.24 (b)	
11	176.0		9a, 9b, 13a
12	77.5		13a, 13b, 18, (17)
13	39.0	2.16 (a), 1.85 (b)	
14	47.6	2.01	13a, 13b, 20, 21
15	176.0		13a, 19, (6b, 21)
17	34.0	1.66	13b, 18
18	7.4	0.85	17
19	31.6	1.79	13b, 14, 20, 21
20	20.0	0.88	21
21	19.9	0.95	20

[a] ^{1}H NMR signal multiplicities and coupling constants (Hz):
Pumiline A: H-2 q ($J = 2.0$), H-3a dq ($J = 16.5, 1.9$), H-3b ddd ($J = 16.5, 3.7, 1.9$), H-5a dd ($J = 10.5, 7.0$), H-5b ddd ($J = 13.5, 10.6, 5.3$), H-6a dddd ($J = 14.3, 13.6, 7.2, 4.2$), H-6b dd ($J = 14.4, 5.3$), H-7 ddd ($J = 5.5, 4.3, 0.9$), H-8 br. m, H-9a d ($J = 12.3$), H-9b dm ($J = 12.3, 1.0$), H-14 d ($J = 10.7$), H-17 s, H-18a d ($J = 4.3$), H-18b d ($J = 4.3$), H-19 dsept ($J = 10.7, 6.5$), H-20 d ($J = 6.4$), H-21 d ($J = 6.6$).
Pumiline B: H-2 q ($J = 1.5$), H-3a br. d ($J = 16.4$), H-3b ddd ($J = 16.4, 5.2, 2.0$), H-5a t ($J = 8.0$), H-5b ddd ($J = 12.5, 9.4, 5.3$), H-6a dddd ($J = 14.1, 12.3, 7.1, 4.3$), H-6b ddt ($J = 13.8, 5.3, 1.4$), H-7 td ($J = 4.6, 1.3$), H-8 br. "s", H-9a d ($J = 11.8$), H-9b dm ($J = 11.8, 0.7$), H-13a dd (J = 14.9, 8.2), H-13b dd (J = 14.9, 1.1), H-14 td ($J = 8.4, 1.1$), H-17 s, H-18 dsept ($J = 8.5, 6.7$), H-19 d ($J = 6.6$), H-20 d ($J = 6.7$).
Pumiline C: H-2 br. "d" ($J > 1.6$), H-6b m, H-7 t ($J = 4.6$), H-9a d ($J = 12.1$), H-9b d ($J = 12.1$), H-13a dd ($J = 14.9, 8.0$), H-13b d ($J = 15.0$), H-14 m, H-17 m, H-18 t ($J = 7.5$), H-20 d ($J = 6.5$), H-21 d ($J = 6.7$).

Identification of Pumiline C (Table 3, Figure 1): This compound could not be isolated in a pure state, and its amount was insufficient to obtain a direct ^{13}C NMR spectrum in a reasonable amount of time. However, it was possible to record ^{1}H,^{13}C-HSQC, and -HMBC spectra. From these, the ^{1}H and ^{13}C chemical shifts were extracted. A number of ^{1}H signals were identified in the one-dimensional proton NMR spectrum. The structure of pumiline C followed from the superposition of its HSQC spectrum upon that of pumiline B. It turned out that pumiline C is a homologue of pumiline B and has an ethyl group connected to C-12 instead of a methyl group. Apart from the replacement of the methyl signal by the ethyl signals, the ^{13}C NMR spectra of pumiline B and C are similar. C-12 in the ethyl compound is deshielded (by 3.0 ppm, β-effect), and C-13 is shielded (by 1.2 ppm, γ-effect) relative to the methyl compound as expected. The average deviation of the remaining ^{13}C chemical shifts between pumiline B and pumiline C is only 0.2 ppm. The similarity of the spectra also speaks for identical overall configurations of these two compounds. The results obtained by the NMR experiments are

confirmed by the similar fragmentation patterns of the mass spectra. Both pumiline B (MW 323) and pumiline C (MW 337) showed a fragment in EI of m/z 308 that could be explained by the loss of a methyl group (M-15) and an ethyl group (MW-29), respectively.

Pumilines A–C are the major macrocyclic PAs in *Crotalaria pumila* (Table 4). They are frequently accompanied by trace amounts of two unknown, related PAs (PA-X1 and PA-X2), which because of their low abundance (generally $<5\%$) could not be isolated in substantial amounts. The PA profiles of *C. pumila* populations from three different locations were established (Table 4). The data indicate great variation in PA composition and concentration among the populations. Within the plants, there are striking differences in the PA profiles of the various organs. In two populations, the simple necine epoxide, subulacine, is the major alkaloid in leaves, whereas the pumilines are dominant in the seeds. The third population (Gardner Canyon) is almost devoid of macrocyclic PAs and contains in leaves and seeds almost exclusively subulacine, with more than 85% relative abundance. Interestingly, the simple necine base supinidine is detectable in variable but substantial amounts in almost all plant organs, especially the seeds, although no supinidine esters are detected in *C. pumila*. Total amounts of PAs are also variable among populations and tissues. The highest levels up to almost 0.6% are reached in flowers and seeds.

In roots, leaves, flowers, and pericarp, PAs are present predominantly if not exclusively as *N*-oxides (Table 5); levels of up to 25% free base in some samples may be explained by spontaneous reduction, which may easily occur postmortem during preservation and preparation of harvested plant samples (Hartmann and Toppel, 1987). In contrast, seeds contain PAs exclusively as free bases. This corroborates previous reports, e.g., seeds of the African species *C. scassellatii*, which also contain PAs exclusively as free bases. These are rapidly *N*-oxidized during germination (Toppel et al., 1988; Chang and Hartmann, 1998).

Pyrrolizidine Alkaloids (PAs) in Larvae and Adults of Estigmene acrea. The PA profiles were qualitatively and quantitatively analyzed in early and late instars reared on an artificial diet. The caterpillars always had the free choice between PA-free diet and the same diet mixed with a *C. pumila* powder. The alkaloid concentration of the PA diet was 0.018% (dry weight basis). A number of larvae were allowed to pupate and develop into adults, which were analyzed in the same way as the larvae. The results for larvae and representative adult individuals in comparison to the PA composition of the diet are listed in Table 6. With the exception of subulacine, which was never detected in insect extracts, all PAs in the diet are sequestered by larvae and transferred into the adult stage. As in the diet, pumilines A and B are always the major alkaloids. Trace amounts of the necine base retronecine, although not present in the diet, were detected particularly in larvae, indicating some degradation of the ingested macrocyclic ester alkaloids.

TABLE 4. PYRROLIZIDINE ALKALOIDS IN VARIOUS ORGANS OF *Crotalaria pumila* POPULATIONS COLLECTED AT THREE DIFFERENT FIELD SITES IN SOUTHEASTERN ARIZONA

			Relative abundance (%)											
			Montosa Canyon			Gardner Canyon			Arivaca Creek Buenos Aires Wildlife Refuge					
Alkaloid	RI (ZB-1)	M^+ m/z	Leaves	Flowers	Pods & seeds	Leaves	Pods & seeds	Roots[a]	Leaves	Stems	Flowers	Pods mature	Seeds young	Seeds mature
Supinidine	1250	139	9	4	5	1	6		tr		6	11	20	26
Subulacine	1282	155	77	70	22	96	87	2	42	tr	14	50	2	2
Pumiline B	2172	323	6	10	32			36	28	tr	34	15	33	31
PA-X1	2206	335		1	1			4			4			2
Pumiline C	2266	337		2	11			31	15		20	9	18	15
PA-X2	2274	339?	2	3			7							
Pumiline A	2370	351	10	10	29	3		25	15		21	16	20	24
Total PA (mg · g^{-1} dry weight):			1.91	5.83	2.75	0.65	0.93	0.4	0.2	tr	1.0	0,6	5.6	2.5

Note. RI: retention index.
[a] From a seed grown greenhouse plant.

TABLE 5. PROPORTION OF TOTAL PYRROLIZIDINE ALKALOIDS IN THE VARIOUS TISSUES OF *Crotalaria pumila* THAT ARE PRESENT AS *N*-OXIDES

	Percentage of total alkaloid present as *N*-oxide				
Alkaloid	Roots	Leaves	Flowers	Pods (without seeds)	Seeds
Supinidine	100	100	>95	100	<5
Subulacine	100	>80	>90	100	nd
Pumiline B	>90	>75	>90	>99	0
PA-X1	100	nd	>80	nd	0
Pumiline C	>90	>75	>80	>99	0
Pumiline A	>90	>75	>80	>99	0

Note. nd, not detected.

In addition to the sequestered *C. pumila* alkaloids, a number of new PAs were identified that are not present in the larval PA diet. These compounds were identified as esters of supinidine, present in the plant diet as free necine base (Table 6) and esters of retronecine, the common necine base of the pumilines. These alkaloids are formed by the insects and are called "insect PAs" (see for review Hartmann, 1999; Hartmann and Ober, 2000). Supinidine was found esterified with 2-hydroxy-3-methylbutyric acid and 2-hydroxy-3-methylpentanoic acid. Both esters are novel alkaloids named estigmines A and B, respectively. The corresponding retronecine esters were named creatonotine A and B (Figure 2). Creatonotine B is identical with the compound already known as creatonotine from adults of the East Asian arctiid *Creatonotos transiens* (Hartmann et al., 1990; Schulz et al., 1993). Creatonotine A was identified recently in PA sequestering leaf-beetles of the genus *Platyphora* (Hartmann et al., 2001; Pasteels et al., 2001). Isocreatonotines A and B are the respective esters of retronecine at C-7. The identification of these alkaloids as monoesters of supinidine and retronecine was achieved by their RI-values, molecular masses, and mass fragmentation patterns of the necine bases (Witte et al., 1993), and identification of the aliphatic 2-hydroxy acids in crude PA extracts following on-line transesterification with trimethylsulfoniumhydroxid in comparison to the authentic hydroxy acids.

Insect PAs may account for 40–70% of total PAs recovered from insects. However, occasionally we observed individuals that contain almost no insect PAs (Table 6, adult 4). Estigmine B and creatonotine B were the dominant insect PAs. The total amount of PAs stored in larvae and adults varies among individuals and ranges from 0.01 to 0.06% dry weight.

Pyrrolizidine Alkaloids (PAs) in Larvae and Adults of Grammia geneura. The PAs of larvae and adults of *G. geneura* were analyzed qualitatively and quantitatively. All individuals originated from a field population collected mainly from

TABLE 6. PYRROLIZIDINE ALKALOIDS IDENTIFIED FROM REPRESENTATIVE LARVAE AND ADULTS OF *Estigmene acrea*

Alkaloid	RI (ZB1)	$[M^+]$ m/z	Relative abundance (%)						
			Young larvae fourth instar $N = 14$	Old larvae last instar $N = 5$	Adult 1	Adult 2	Adult 3	Adult 4	Larval diet
Plant acquired									
Supinidine	1256	139	4	2	2	4	—	20	19
Subulacine	1282	155	—	—	—	—	—	—	13
Retronecine	1425	155	3	1	—	3	—	—	—
Pumiline B	2172	323	23	17	11	12	13	18	31
PA-X	2228	335	1	1	tr	tr	—	4	2
Pumiline C	2266	337	12	8	5	4	8	16	13
Pumiline A	2370	351	15	13	10	13	16	39	22
Sum (% of total PAs)			58	42	28	36	37	97	100
Insect synthesized									
Estigmine A	1736	239	1	4	4	4	—	—	
Estigmine B	1835	253	6	18	25	8	11	3	
Isocreatonotine A	1864	255	1	1	4	9	—	—	
Creatonotine A	1886	255	5	5	tr	3	—	—	
Isocreatonotine B	1962[a]	269	9	7	8	12	13	—	
Creatonotine B	1985[a]	269	20	22	31	27	39	—	
Sum (% of total PAs)			42	58	72	64	63	3	
Total PAs (mg · g^{-1} dry weight):			0.50	1.00	0.315	0.615	0.098	0.177	0.018

Note. RI: retention index.
[a] Occurrence often as double peak indicating the presence of stereoisomers (identical mass fragmentation).

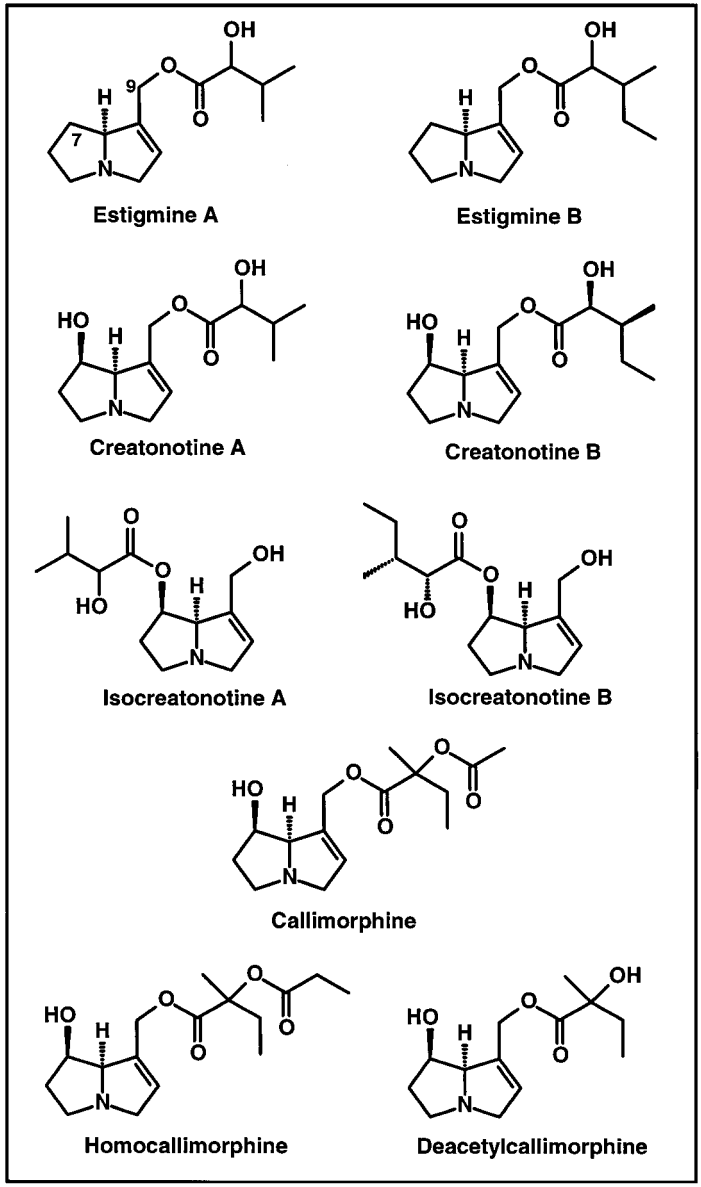

FIG. 2. Structural formulae of the insect PAs identified from *Estigmene acrea* and *Grammia geneura*. In the insects, all PAs are stored as *N*-oxides.

S. longilobus as penultimate or final instars. Larvae were further reared in the laboratory and offered a mixture of two acceptable PA-free food-plants, *Malva* and *Ambrosia,* and as PA source, *S. longilobus.* Table 7 shows the PA profiles of four selected representative larvae, all of them final instars, and of four representative adults. The PA pattern established for both larvae and adults is qualitatively identical with the PA pattern of the larval PA source (see Table 2). In larvae, the plant-acquired alkaloids accounted for almost 100% of total PAs; no or only traces of insect PAs could be detected. By contrast, adult individuals showed, in addition to the plant acquired PAs, a rich pattern of insect PAs that accounts for 17–37% of total PAs found in the four analyzed representative individuals (Table 7). The dominant insect PA in *G. geneura* is callimorphine (Figure 2). Callimorphine and isocallimorphine were identified by GC-MS in comparison to reference data and identification of 2-hydroxy-2-methylbutyric acid in crude PA extracts after on-line transesterification with trimethylsulfoniumhydroxid in comparison to the authentic 2-hydroxy acid. The two PAs are accompanied by trace amounts of creatonotine B and two new insect PAs that were tentatively identified by GC-MS as deacetylcallimorphine and homocallimorphine. Both display almost the same fragmentation patterns as callimorphine. However, both lack the fragment at m/z 43 (-acetyl), and in homocallimorphine a new fragment at m/z 57 (-propionyl) is present, indicating the deactylated derivative and replacement of an ethyl residue by a propionyl group, respectively.

Total PA concentrations varied considerably among the representative individuals analyzed. They ranged from 0.14 to 1% dry weight and were about one order of magnitude higher than the PA levels found in *E. acrea.*

DISCUSSION

In this work, we identified new PA structures in plants and insects, and demonstrated that the two generalist arctiids sequester diverse PAs from their host plants. Compounds present in the insects reflect those present in the hosts, but additional PAs are found in the insect (insect PAs) that are synthesized from necine bases derived from the acquired PA mixtures. In all cases, insects store the PAs as *N*-oxides.

Plant PAs. Only one of the four species of Boraginaceae, *Plagiobothrys arizonicus,* contained PAs. Small quantities of five known alkaloids were identified, and this is the first PA record for this plant species. Surprisingly, no PAs were found in *Cryptantha barbigera,* since this genus includes many species containing PAs (Stermitz et al., 1993).

Investigation of local populations of *Senecio longilobus* revealed an extension of the known alkaloid pattern for this species and confirmed that there is great variation in alkaloid composition and total PA concentration (Johnson et al., 1985; Ray et al., 1987; Cooper et al., 1996). Particularly high concentrations of

TABLE 7. PYRROLIZIDINE ALKALOIDS IDENTIFIED FROM REPRESENTATIVE LARVAE AND ADULTS OF *Grammia geneura*

			Relative abundance (%)							
Alkaloid	RI (ZB1)	$[M^+]$ m/z	Larva 1	Larva 2	Larva 3	Larva 4	Adult 1	Adult 2	Adult 3	Adult 4
Plant acquired										
Retronecine	1425	155	—	—	tr	—	—	tr	tr	tr
Senecionine	2294	335	19	2	23	12	4	4	tr	4
Seneciphylline	2310	333	18	14	18	12	22	20	29	12
Spartioidine	2346	333	4	8	5	7	11	10	14	5
Integerrimine	2353	335	30	10	30	20	13	23	10	20
Retrorsine	2522	351	25	28	19	35	23	19	18	14
Usaramine	2584	351	4	30	5	9	6	5	8	8
Sum (plant acquired)			>95	>95	>95	100	79	83	79	63
Insect synthesized										
Deacetylcallimorphine[a]	1830	255	—	—	—	—	tr	tr	tr	2
Isocreatonotine B	1962	269	tr	tr	—	—	2	3	—	2
Callimorphine	1964	297	—	—	—	—	16	7	21	28
Creatonotine B	1980	269	tr	2	—	—	3	7	tr	5
Homocallimorphine[a]	2044	311	—	—	—	—	tr	tr	tr	tr
Sum (insect synthesized)			<5	<5	<5	—	21	17	21	37
Total PAs (mg · g^{-1} dry weight)			1.6	1.5	2.8	6.5	10.0	3.9	1.4	2.1

Note. RI: retention index. Final instar larvae were reared on a plant mixture of *Malva parviflora*, *Ambrosia confertifolia,* and *Senecio longilobus.*
[a] Tentatively identified by GC-MS.

the alkaloids were found in foliage from one population. It is not known whether such differences might partly reflect differences in the proportion of young and mature leaves in the plants at different sites, since considerable small scale variation in rainfall in southeastern Arizona leads to local differences in phenology. Pronounced ontogenetic and seasonal variation in PA concentrations in the leaves of *S. longilobus* have been documented—an approximately 10-fold variation was observed in mean alkaloid content over the season (Johnson et al., 1985).

The alkaloid profile of *C. pumila*, which has not been described before, led to the discovery of three novel macrocyclic PAs of the monocrotaline type, named pumilines A–C. The few samples of *C. pumila* that were analyzed indicate great qualitative and quantitative variations in the alkaloid profiles among local populations and within a population among various plant organs (Table 4).

Great quantitative and qualitative variability seems to be a general feature of PAs. It has been reported in detail for *S. vulgaris* and *S. vernalis* (von Borstel et al., 1989) and *S. jacobaea* and *S. erucifolius* (Witte et al., 1992). In *S. jacobaea*, this phenotypic variation was found to be largely attributed to genetic variation (Vrieling et al., 1993), and may be related to the physiology of PA biosynthesis. In many *Senecio* species, senecionine *N*-oxide is synthesized as a common backbone structure that is subsequently structurally diversified by simple one- or two-enzyme steps into the species-specific PA pattern (Hartmann and Dierich, 1998). Since PAs do not show any turnover, even a slight change in expression or activity of the enzymes involved would cause substantial alterations in the PA profile. In some species, structural diversification occurs in an organ-specific manner, creating different, and sometimes distinctive organ-specific PA profiles. It can be noted here in *C. pumila*, for example, in the distribution of the simple necine epoxide subulacine, which dominates in the vegetative organs, and the pumilines, which predominate in the reproductive structures, especially seeds (see Table 4).

Caterpillar Sequestration of PAs. The two caterpillars studied here appear to have broad substrate specificity for uptake, safe storage, and stable maintenance of PAs. *E. acrea* sequester without discrimination all PAs occurring in *C. pumila* except the simple epoxide subulacine, and the same is true for *G. geneura* feeding on *S. longilobus*. All sequestered PAs were also found in adult moths in concentrations similar to those in the larvae, indicating that the alkaloids are not principally in the cuticle and that they survive intact during metamorphosis.

Accumulation and safe maintenance of a broad structural spectrum of protoxic PAs by adapted arctiids is guaranteed by a specific enzyme that catalyzes the conversion of the toxic-free base into its nontoxic *N*-oxide. This enzyme, a mixed function flavin monooxygenase, senecionine *N*-oxygenase, has been purified as a soluble NADPH-dependent enzyme from the hemolymph of larvae of the cinnabar moth, *Tyria jacobaeae* (Lindigkeit et al., 1997). Senecionine *N*-oxygenase converts, with broad substrate specificity, all absorbed sequestered toxic-PA-free bases efficiently into the respective *N*-oxides. The enzyme is highly specific for PAs;

nontoxic PAs, e.g., those lacking the 1,2-double bond in the necine base moiety, as well as other alkaloids or derivatives tested are not substrates. The substrate specificity for all major structural types of toxic PAs indicates that senecionine *N*-oxygenase has been recruited and optimized for its new function in the course of coevolutionary adaptation of arctiids to PA-containing plants. Molecular evidence for this idea has been presented (Naumann et al., 2002). Senecionine *N*-oxygenase has been detected in all PA sequestering arctiids analyzed so far, among them the polyphagous *Arctia caja*, the garden tiger moth, and the East Asian moth *Creatonotos transiens* (Lindigkeit et al., 1997). Since PAs in *E. acrea* and *G. geneura* are stored as *N*-oxides, the enzyme should also be present in these species and guarantee tolerance and maintenance of plant acquired PAs. Generally, in arctiids, any ingested PA *N*-oxide is reduced in the gut and absorbed as free base into the hemolymph where it is detoxified by *N*-oxidation. A previous report on a PA *N*-oxide carrier in the gut of arctiids (Wink and Schneider, 1988) has been disproved (Lindigkeit et al., 1997).

PA Metabolism in Insects. Analysis of the two arctiids not only revealed the presence of PAs absorbed from their natural diets or synthetic diets laced with plant material, it also showed the presence of a rich pattern of insect PAs, including several novel structures (see Figure 2). Insect PAs are esters of acquired necine bases, which are found occasionally in PA-sequestering insects, but are not derived directly from their respective food plants. The first observed insect PA was a "PA metabolite" detected in pupae of *Tyria jacobaeae* (Aplin et al., 1968); it was later structurally elucidated and named callimorphine (Edgar et al., 1980). Callimorphine is synthesized in the insect by esterification of plant-derived retronecine with a necic acid of insect origin (Ehmke et al., 1990). Callimorphine has been detected in a number of arctiid moths (see review Hartmann and Witte, 1995). Other insect PAs with creatonotine and isocreatonotine (now named creatonotines B) as major compounds were identified from adults of *Creatonotos transiens* (Hartmann et al., 1990; Schulz et al., 1993).

In the present study, six new insect PAs were observed that, together with the already known ones, have in common that all necic acids are aliphatic 2-hydroxyacids (see Figure 2). Two of these acids are the respective 2-hydroxy analogs of valine and isoleucine, and the third one is 2-hydroxy-2-methylbutyric acid, which in *G. geneura* is present esterified to retronecine (named deacetylcallimorphine), as well as the 2-*O*-acetyl derivative (already known as callimorphine) and the 2-*O*-propionyl derivative (named homocallimorphine). In *E. acrea*, the presence of the two supinidine esters, estigmines A and B, suggests esterification of the free supinidine ingested with the larval diet. The respective retronecine esters, now named creatonotines A and B, indicate partial degradation of the ingested pumilines for providing the required free retronecine that is not present in the diet. This must also be true for *G. geneura*, where the senecionine-type PAs should be the precursors for the required retronecine.

In both *E. acrea* and *G. geneura*, the insect PAs represent an important proportion of total PAs, i.e., up to more than 70% in *E. acrea* and more than 35% in *G. geneura*. The relevance of insect PA synthesis is not yet understood, but it may represent a salvage mechanism for recycling of a necine base into an ester alkaloid with defensive activity. It is also not known why there are a few individuals that are almost devoid of insect PAs. All arctiids that produce the mating pheromone hydroxydanaidal must be able to degrade the necic acid moiety of the various structural types of PAs. This has been repeatedly indirectly demonstrated (Boppré, 1986; Schneider, 1987; Krasnoff and Roelofs, 1989). The mechanism by which a PA is converted into a pheromone has been shown for the degradation of heliotrine (Schulz et al., 1993; Schulz, 1998). In this process, retronecine appears not to be a direct precursor, but is suggested to be incorporated into the pheromone via the insect PA creatonotine (now creatonotine B). Since *T. jacobaeae* and most probably also *G. geneura* do not synthesize pheromones but produce insect PAs, an essential role of insect PAs as precursors of pheromone biosynthesis appears unlikely.

A significant difference between the two arctiids is that *E. acrea* produces the insect PAs already in the larval stage, while *G. geneura* larvae do not contain insect PAs. The latter case is comparable to *Tyria jacobaeae*, where the pre-pupa was found to be the first developmental stage that contains insect PAs (Ehmke et al., 1990).

Formation of insect PAs in arctiids has an intriguing parallel in PA-adapted leaf beetles of the genus *Platyphora*, which sequester PAs of the lycopsamine type (Pasteels et al., 2001). These beetles synthesize exactly the same type of insect PAs. Like arctiids, they produce retronecine esters with 2-hydroxy analogs of aliphatic amino acids (Hartmann et al., 2001, 2003). Aliphatic 2-hydroxy acids that are commonly found in the autogenously synthesized insect necine esters of arctiids and leaf beetles are rarely found in other insects. The only example we are aware of is the occurrence of 2-hydroxy-3-methylbutyric acid as a minor constituent in exocrine secretions of gypsy moth caterpillars (Aldrich et al., 1997). The formation of the same type of insect PAs in arctiids and leaf beetles is an exceptional example of convergent biochemistry, raising a number of intriguing questions.

Adaptation of Generalists to PA Use. Like other arctiids, *E. acrea* and *G. geneura* must use a number of specific physiological mechanisms to ingest, detoxify, accumulate, recycle, and transfer plant-acquired PAs. This biochemical specificity is corroborated and complemented by the recently established sensory specificity that allows caterpillars to recognize their PA sources. It is well known that PAs are behavioral phagostimulants for larvae of adapted arctiids (Boppré, 1986; Schneider, 1987; Bogner and Eisner, 1991), indicating that larvae possess taste receptors for their detection. Taste receptor cells are present in both the lateral and medial galeal styloconic sensilla of larvae of *E. acrea*. One receptor cell (in the lateral sensillum) is sensitive to concentrations down to at least 10^{-9} M; it

also responds to other secondary metabolites like ouabain as well as amino acids, but with a much higher threshold (Bernays et al., 2002a). The second receptor cell (in the medial sensillum) also responds to PAs, but appears to be specifically dedicated to the perception of PAs; no other compound was found to affect this cell (Bernays et al., 2002b). Neurons that respond with similar high sensitivity to PAs were detected in *G. geneura* (Bernays et al., 2002b).

In both of the generalist arctiids, sensitivity levels were equal to those of the larvae of the monophagous arctiids *Utetheisa ornatrix* (Bernays et al., 2003a) and *Tyria jacobaeae* (Bernays et al., 2004). The sensitive sensory recognition of PAs may be of special importance for caterpillars of polyphagous arctiids like *E. acrea* and *G. geneura*. It enables them to identify their PA plants and start feeding and ingesting PAs. However, both *E. acrea* and *G. geneura* survive poorly if they are reared on *Senecio* or *Crotalaria* alone (E. A. Bernays, M. S. Singer, and D. Rodrigues, unpublished results). Recently, it was found that extensive feeding of *E. acrea* on plant material or a diet rich in PAs causes a loss in the response of the PA-sensitive cell in the lateral PA sensillum (Bernays et al., 2003b). The effect was also achieved by injection of a PA into the hemolymph, indicating a physiological feedback between PA load in the hemolymph (or body) and sensory activity. The short-lived loss of sensory response to PAs is paralleled by loss of behavioral acceptance of PA plants, and apparently provides a mechanism to enable the caterpillar to utilize a PA plant just as a source for its alkaloidal pharmacon and move on to find other favorable food plants. Thus, even among these generalist arctiids, specialized physiological mechanisms for finding, sequestering, altering, and utilizing PAs appear to be as profound as in specialist species.

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THE ROLE OF METHYL SALICYLATE IN PREY SEARCHING BEHAVIOR OF THE PREDATORY MITE *Phytoseiulus persimilis*

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Abstract—Many carnivorous arthropods use herbivore-induced plant volatiles to locate their prey. These plant volatiles are blends of up to hundreds of compounds. It is often unknown which compounds in such a complex volatile blend represent the signal to the foraging carnivore. We studied the role of methyl salicylate (MeSA) as part of the volatile blend in the foraging behavior of the predatory mite *Phytoseiulus persimilis* by using a Y-tube olfactometer. MeSA is one of the compounds released by lima bean, infested with *Tetranychus urticae*—a prey species of the predatory mite. MeSA attracted satiated predatory mites in a dose-dependent way with optimum attraction at a dose of 0.2 μg. Predatory mites did not discriminate between a prey-induced lima bean volatile blend (that contains MeSA) and a prey-induced volatile blend to which an extra amount of synthetic MeSA had been added. However, they preferred a MeSA-containing volatile blend (induced by *T. urticae*) to an otherwise similar but MeSA-free blend (induced by jasmonic acid). Adding synthetic MeSA to the MeSA-free blend significantly increased the mites' choice for this odor, suggesting an important role for MeSA. This study is a new step toward unraveling the role of herbivore-induced plant volatiles in the foraging behavior of predatory arthropods.

Key Words—Plant–carnivore interactions, herbivore-induced plant volatiles, tritrophic interactions, variation, jasmonic acid, specificity, methyl salicylate.

INTRODUCTION

Animals base their foraging decisions on information from their environment. One would expect animals in search of food to focus on the parts of the available

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information that are associated with their food, to maximize the efficiency of information use (Bernays and Wcislo, 1994; Bernays, 2001). However, it is often unknown which parts of the information contain the relevant signals for a foraging animal (see for example Dicke, 1999). We studied this issue for a predatory mite that uses herbivore-induced plant volatiles to locate its herbivorous prey.

Most prey species are inconspicuous: they are under selection to minimize the information that they emit to reduce the risk of predation. Consequently, various carnivorous animals use information that is not emitted by their prey but instead by their prey's direct environment. A well-explored example of this phenomenon is the use of information from the prey's food plant by natural enemies of arthropod herbivores (Vet and Dicke, 1992; Paré and Tumlinson, 1999). Although plants can produce easily detectable and reliable information about the presence of herbivorous arthropods, the composition of herbivore-induced plant volatile blends can be highly variable. Many factors influence the composition: plant species, plant cultivar, leaf age, and plant tissue, but also herbivore species or even developmental stage (e.g., Takabayashi et al., 1994b, 1995; De Moraes et al., 1998; Turlings et al., 1998; Gouinguené et al., 2001). A pathogen infection in addition to herbivore damage may also affect volatile blend composition (Cardoza et al., 2002). Furthermore, environmental conditions that affect the plant's physiology, such as light and water, contribute to variation in volatile production (Takabayashi et al., 1994a; Gouinguené and Turlings, 2002).

Given the large variation in available information, the question arises which parts of the volatile blends are used by the carnivores to detect the presence of their prey? Little is known about how carnivores perceive and interpret volatile blend composition and its variation (Dicke, 1999; Dicke and Vet, 1999; Turlings and Fritzsche, 1999; Vet, 1999a). Because of the large overlap in herbivore-induced volatiles between different plant-herbivore complexes (for example, Turlings et al., 1993; Paré and Tumlinson, 1999; Van den Boom et al., in press), it is generally assumed that most carnivores do not use a single key-compound to detect prey. Indeed, carnivores perceive and respond to more than one compound of herbivore-induced plant volatile blends (Dicke et al., 1990; De Bruyne et al., 1991; Smid et al., 2002). On the other hand, it is also not expected that all parts of a volatile blend contain relevant information for a searching carnivore, because not all variation in blend composition is related to the presence of the prey herbivore.

Several studies have addressed the question of which compounds in a complex odor blend cause the attractiveness of a blend to carnivorous arthropods (Dicke et al., 1990; Turlings et al., 1991; Scutareanu et al., 1997). A good way to narrow the range of potentially active compounds in a blend is by gas chromatography coupled with electroantennography (GC-EAG) (e.g., Du et al., 1998; Weissbecker et al., 2000). Using this technique, volatiles that are perceived by the sensory system of the arthropod can be identified. However, activity of compounds at the sensory level is not always directly correlated to behavioral activity (Li et al., 1991). The

ecological significance of potentially interesting compounds should, therefore, always be evaluated in behavioral set-ups.

In a field situation, a foraging carnivore will have to make choices among different volatile blends, e.g., induced by different herbivore species. The question of which differences between of blends enable carnivores to make such a choice has not yet been answered (Vet, 1999a, b; Dicke and Van Loon, 2000). Using a synthetic mimic of a natural volatile blend to answer this question is difficult because the sensitivity of analytical equipment is lower than the sensitivity of predatory arthropods (Pickett et al., 1998), and not all herbivore-induced compounds have been identified. We avoided this problem by using two plant volatile blends of known similar composition, and between which a carnivore can discriminate. By restoring one volatile blend with the compounds that it misses compared to the other volatile blend, the role of the compounds in enabling the carnivore to discriminate can be identified. To obtain plant volatile blends with a partially different composition, different herbivore species or chemical elicitors such as plant hormones can be used.

We studied the attraction of the predatory mite *Phytoseiulus persimilis* to lima beans infested with the herbivorous mite *Tetranychus urticae*. *P. persimilis* can use herbivore-induced plant volatiles to locate prey (e.g., Sabelis and Van de Baan, 1983; Dicke et al., 1990; Drukker et al., 2000). Because the spider mite *T. urticae* is a highly polyphagous herbivore (Helle and Sabelis, 1985), *P. persimilis* is potentially confronted with a large variation in herbivore-induced blends.

We specifically investigated the influence of both quantitative and qualitative differences in the amount of one compound of the herbivore-induced volatile blend of lima beans—methyl salicylate (MeSA)—on the foraging behavior of *P. persimilis*. Several studies have presented data that indicate a role for MeSA in foraging of *P. persimilis*. First, MeSA is attractive to *P. persimilis* when offered as a single compound (Dicke et al., 1990). Second, *P. persimilis* prefers the volatiles from *T. urticae*-induced lima bean to the volatiles from jasmonic acid (JA)-induced lima bean, and the absence of MeSA in the latter blend is one of the most important differences between the two (Dicke et al., 1999).

In this article, we study the predatory mites' responses to: (1) Different doses of MeSA. (2) Two natural *T. urticae*-induced lima bean volatile blends, one of which was combined with additional synthetic MeSA. (3) A natural volatile blend (*T. urticae*-infested lima bean), an incomplete blend (JA-induced lima bean), and an incomplete, but restored blend (JA-induced lima bean plus MeSA) vs. uninfested lima bean volatiles. (4) The natural *T. urticae*-induced lima bean volatile blend vs. the incomplete JA-induced blend, which was or was not restored with synthetic MeSA.

METHODS AND MATERIALS

Plants and Mites. Lima bean plants (*Phaseolus lunatus* L., cv Sieva) were reared in a greenhouse ($25 \pm 5^{\circ}$C, 50–70% R.H., photoperiod 16L:8D) and used

in experiments when the primary leaves had unfolded (10–15 days after sowing). A culture of the two-spotted spider mite, *Tetranychus urticae* Koch, was kept on lima bean plants under greenhouse conditions. The predatory mites, *Phytoseiulus persimilis* Athias-Henriot, were originally obtained from Entocare CV, The Netherlands. In our laboratory, they were reared on lima bean leaves infested with *T. urticae* in Petri dishes (9-cm in diameter) in a climate room ($23 \pm 1^{\circ}$C, 50–70% R.H., 16L:8D). In all experiments, we used mated adult females, 1–2 days after the final molt. Females were kept individually in Eppendorf vials for 2–5 hr (satiated) or 22–26 hr (starved) before an experiment at $23 \pm 1^{\circ}$C. We used both satiated and starved predatory mites because starvation level may affect mites' searching behavior (Dicke et al., 1998; Shimoda and Dicke, 2000).

Y-Tube Olfactometer. In all experiments, the response of the predatory mites was tested in a Y-tube olfactometer (Takabayashi and Dicke, 1992). Air filtered through activated charcoal was blown through both arms of the olfactometer at 4 l/min. Mites were individually introduced at the basal end of the Y-tube. They were placed on a metal wire running through the center of the glass tube. The predator's behavior was observed, and a choice was recorded when the predator passed a grid halfway up one of the arms of the Y-tube. A "no choice" was recorded when the predator did not reach the grid within 5 min. Odor sources were interchanged between the arms of the Y-tube after every set of five mites to compensate for an influence of unforeseen asymmetric aspects of the set-up. Each experiment was repeated on four to six different days with new groups of predatory mites and new odor sources per day. In experiments where we compared the responses of satiated and starved mites, individuals of the two groups were tested alternately with the same odor sources. All olfactometer experiments were conducted at $21 \pm 2^{\circ}$C.

Response to Methyl Salicylate. Methyl salicylate (MeSA, Merck, 99% pure) was diluted in n-hexane (Merck, pro-analysis). In all experiments, 0.1 ml of the diluted MeSA was applied on a piece of filter paper (15 cm^2) and placed in the last section of one of the arms of the Y-tube olfactometer. A filter paper with 0.1-ml hexane was placed at the same position in the other arm as a control. A predatory mite was introduced into the olfactometer after 30 to 60 sec when most of the solvent had evaporated. New filter papers with MeSA or hexane were used for each mite. For every dose of MeSA (0.002 μg, 0.02 μg, 0.2 μg, 2 μg, 20 μg, and 200-μg MeSA dissolved in 0.1-ml hexane), 19–30 satiated mites were tested per experimental day, and this was repeated on 5 or 6 days. Different doses of MeSA were tested on different days. To compare responses of satiated and starved mites to 0.2 μg MeSA, we tested both groups in a separate experiment. Twenty to 27 females of each group were tested alternately on four different days.

Quantitative Differences in Amount of Methyl Salicylate. In this experiment, we studied the effect of varying the relative amount of MeSA in a volatile blend on the behavior of predatory mites. This was done by adding MeSA to a natural,

MeSA-containing, volatile blend emitted by spider mite-infested lima bean leaves. Primary leaves were detached, individually placed with their petiole in glass vials filled with water (15 ml), and infested with 50 adult spider mite females per leaf. The leaves were incubated in a plastic cage in a climate room for 3 days at 23 $\pm$ 1°C, 50–70% R.H., and 16L:8D. One odor source consisted of four *T. urticae*-infested lima bean leaves; the alternative odor source consisted of the other infested primary leaves from the same four plants. In this way, we standardized the two sets of infested leaves to minimize differences between the two odor sources. Each odor source was placed into a glass jar (2 l) and connected to the Y-tube olfactometer with plastic tubing. Although we expected the two sets of infested leaves to be equally attractive, we first determined the response of 10–22 *P. persimilis* females to both odor sources. After the relative attraction of both odor sources was determined, a filter paper with 0.1-ml MeSA solution was placed in the olfactometer arm downwind of the set of infested leaves that had attracted 50% or more of the mites in the previous relative attraction test. It should be noted that the lima bean leaves were not exposed to the MeSA so MeSA cannot have affected the volatile emission by these leaves. A filter paper with 0.1-ml hexane was placed at the same position in the other arm of the Y-tube. New filter papers with MeSA and hexane were used for every mite. We observed the choices of a new set of 10–21 mites to the volatiles from *T. urticae*-infested leaves vs. the combination of *T. urticae*-infested leaves and synthetic MeSA. The influence of different amounts of synthetic MeSA on the choice of satiated predators between two natural volatile blends was studied: 0.2 μg, 2 μg, 20 μg, and 200 μg. Starved mites were tested only at a MeSA dose of 0.2 μg. The experiments were repeated on four different days for each dose of MeSA.

Qualitative Differences in Amount of Methyl Salicylate. To obtain odor sources that differed qualitatively in the presence of MeSA, we used the plant hormone jasmonic acid (JA). Lima bean plants incubated with JA emit a volatile blend that is similar to the volatile blend released by *T. urticae*-induced lima bean, except for the presence of MeSA (Dicke et al., 1999). The following four comparisons were made to investigate the importance of a qualitative difference in the amount of MeSA: (1) JA-induced lima bean vs. clean lima bean: Plants were cut at soil level and placed with their stem into a glass vial containing a 15-ml solution of jasmonic acid (1 mM, JA, Sigma-Aldrich) in water. They were incubated for two days in a plastic cage in a climate room (23 $\pm$ 1°C, 50–70% R.H., 16L:8D). Air was sucked from the cage by house vacuum. Clean lima bean plants, incubated in the same way in vials with water only, served as the controls. Nine leaves were used per odor source. The experiment was repeated on 4 days with 20–25 satiated and 21–23 starved predatory mites per day. (2) JA-induced lima bean plus synthetic MeSA vs. clean lima bean: One odor source consisted of nine JA-induced lima bean leaves (see above) in a glass jar and a filter paper with 0.2-μg MeSA in the last section of the Y-tube (i.e., downwind of the leaves, to avoid the leaves being

exposed to MeSA). The control odor source consisted of nine uninfested lima bean leaves in a glass jar and a filter paper with 0.1-ml hexane downwind in the olfactometer-arm. Nineteen to 21 satiated and 19–21 starved mites were tested per day, and the experiment was repeated on 4 days. (3) *T. urticae*-induced lima bean vs. clean lima bean: Odor sources consisted of four lima bean leaves, each infested with 50 *T. urticae* females, and four uninfested leaves. Leaves were incubated as described above in the section "Quantitative differences in amount of methyl salicylate." The experiment was repeated on 4 days with 20–23 satiated and 14–21 starved mites per day. (4) *T. urticae*-induced lima bean vs. JA-induced lima bean plus synthetic MeSA: Plants were cut at soil level and placed with their stem in a glass vial with JA dissolved in water (1 mM, 15 ml). They were incubated for two days in a plastic cage in a climate room ($23 \pm 1^{\circ}$C, 50–70% R.H., 16L:8D). Plants infested with 50 *T. urticae* females per leaf were incubated in the same way in water only. Nine leaves were used per odor source. After testing the choice of about 10 predatory mites in the Y-tube olfactometer, a filter paper with MeSA (0.2 μg in 0.1-ml hexane) was placed into the olfactometer arm, downwind of the JA-induced leaves. A filter paper with hexane was placed into the other arm as a control. Then, the response of a new set of 20–22 mites to the volatiles from *T. urticae*-infested leaves plus hexane vs. the volatiles from JA-induced leaves plus MeSA was tested. Subsequently, the last sections of both olfactometer arms were replaced by clean parts to exclude effects of any MeSA or hexane left on these parts. Then, the response to *T. urticae*-induced volatiles vs. JA-induced volatiles was tested for another 10 mites. Responses of the two groups of 10 mites that were used to determine the predator's choice between JA-induced and *T. urticae*-induced volatiles, were not different (2×2 contingency table, $\alpha = 0.05$) and, thus, the summed results of these two groups are presented. Satiated and starved mites were tested in experiments carried out on different days. This experiment was repeated on four different days per starvation level.

Data Analysis. Contingency table analysis ($N \times 2$ contingency tables, where N = number of experimental days) showed that results from replicate tests, done on different days with different batches of odor sources and different sets of predators, do not differ significantly. Therefore, the data from different experimental days were pooled for each experiment. A binomial test was used to analyze the predator's choices in the olfactometer (i.e., a difference from a 50:50 distribution between the two odor sources) for all experiments. We used a two-sided binomial test for all experiments except when uninfested leaves were tested against treated leaves, in which case we used a one-sided binomial test (expecting a preference for the treated bean leaves). Predators that did not make a choice were excluded from the statistical analysis. Overall, the percentage of satiated predators that did not make a choice was 11%, while less than 4% of the starved mites did not make a choice.

The response of satiated mites to different doses of MeSA was analyzed by fitting a generalized linear model (GLM), using the binomial distribution and

logit-link-function with log-dose and square of log-dose as predicting factors (Crawley, 1993). To test the effect of starvation level on the responses of mites to MeSA, JA-induced lima bean volatiles (plus MeSA), and *T. urticae*-induced lima bean volatiles, we fitted a GLM with replicate and starvation level as predicting factors. The same procedure was used to test the effect of adding synthetic MeSA on the choice of predators between two *T. urticae*-induced volatile blends or between JA-induced and *T. urticae*-induced volatiles (predicting factors: replicate + odor source).

RESULTS

Response to Methyl Salicylate. Methyl salicylate offered as a single compound was attractive to satiated *P. persimilis* females (Figure 1). Although a low dose of 0.002 μg did not attract *P. persimilis* ($P = 0.21$, binomial test), doses from 0.02 μg to 20 μg attracted the predatory mites ($P \leq 0.018$). The mites were

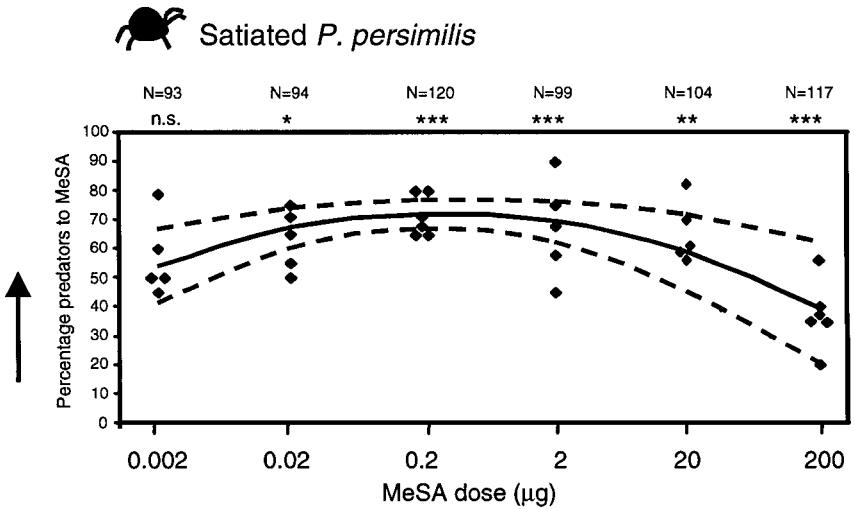

FIG. 1. Response of female *Phytoseiulus persimilis* to different amounts of MeSA in the Y-tube olfactometer vs. control (hexane). Each square represents the percentage of satiated predatory mites choosing MeSA in one replicate experiment with about 20 females. The solid curve shows the predicted values of the generalized linear model (see Methods and Materials) for the attraction of mites to different doses of MeSA; the dotted curves indicate the 95-confidence limits of the predicted values of the model. The total number of mites that made a choice is given for each dose of MeSA. Responses to each different dose of MeSA were statistically analyzed with a one-sided binomial test (ns $P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

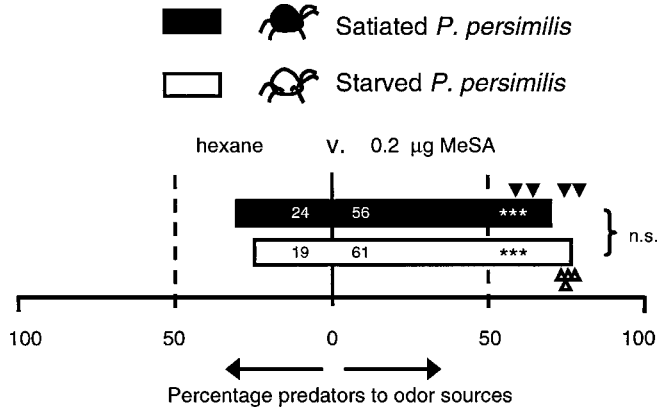

FIG. 2. Response of satiated (black bar) and starved (white bar) *Phytoseiulus persimilis* to 0.2-μg MeSA. The numbers in the bars are the absolute numbers of predatory mites that made a choice for MeSA or the control. The triangles represent the percentage of predatory mites in each replicate experiment that was attracted to MeSA (filled triangles: satiated predatory mites, open triangles: starved predatory mites). Choices between odor sources were analyzed with a two-sided binomial test; effect of starvation level was analyzed with a GLM (ns $P > 0.05$; $^{***}P < 0.001$).

repelled by the highest dose of MeSA (200 μg, $P < 0.001$). The maximum attraction to MeSA was found at 0.2 μg, where on average 72% of the mites were attracted. The percentage of mites attracted to MeSA was dose-dependent. Both log-dose and square-log-dose were significant as factors predicting attraction ($P < 0.001$). The dose-response curve as described by the model is shown in Figure 1.

Starved predatory mites were also attracted to 0.2-μg MeSA (Figure 2, $P < 0.001$, binomial test). Their response was not significantly different from the response of satiated predatory mites to 0.2-μg MeSA ($P = 0.37$, GLM).

Quantitative Differences in Amount of Methyl Salicylate. When satiated predatory mites were offered a choice between volatiles from two sets of *T. urticae*-infested lima bean leaves—one having an additional amount of synthetic MeSA, and the other not, the extra amount of MeSA did not affect the attraction of *P. persimilis* to the natural volatile blend, except when the highest repellent dose of MeSA (200 μg) was added (Figure 3). In the latter case, the average attraction toward the volatiles from the most attractive set of *T. urticae*-infested lima bean leaves was 61%, while after adding 200-μg MeSA, the attraction dropped to 31% ($P = 0.003$, GLM).

Adding 0.2-μg MeSA to one of two sets of *T. urticae*-infested leaves did not influence the response of starved *P. persimilis* to the volatiles from these two sets of infested leaves (Figure 3, $P = 0.32$, GLM).

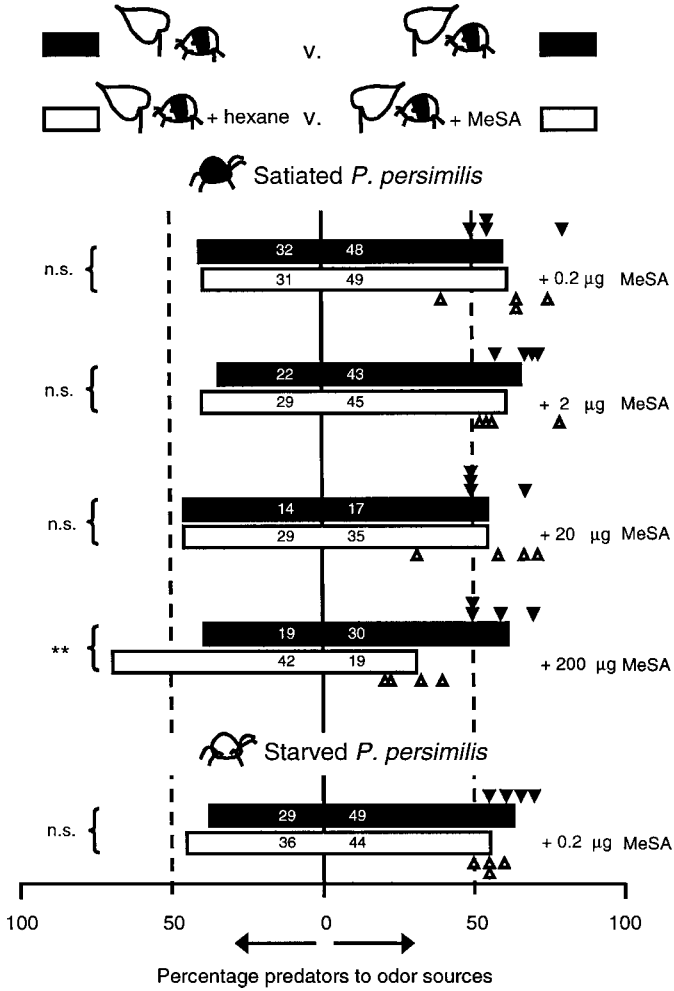

FIG. 3. Influence of relative amount of MeSA on the attraction of satiated and starved *Phytoseiulus persimilis* to the volatiles from *Tetranychus urticae*-infested lima bean leaves. Black bars present the percentage of predatory mites choosing between the volatiles from two equal sets of *T. urticae*-infested lima bean leaves. The white bars present the responses of predators to the same sets of leaves when synthetic MeSA had been added to one of them (downwind; 0.2 μg, 2 μg, 20 μg, 200 μg). Numbers in bars are the absolute numbers of predatory mites responding to each odor source. The triangles indicate the percentage of predatory mites in each replicate experiment that was attracted to *T. urticae*-infested lima bean leaves (filled triangles) or to the combination of *T. urticae*-infested lima bean leaves plus MeSA (open triangles). The effect of adding synthetic MeSA was analyzed with a GLM (ns $P > 0.05$; $^{**}P < 0.01$).

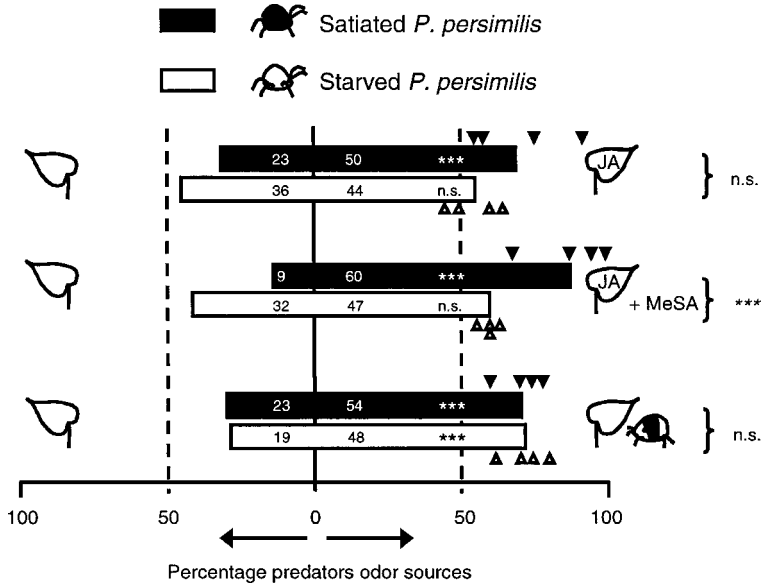

FIG. 4. Effect of a qualitative difference in amount of MeSA on the responses of satiated (black bars) and starved (white bars) *Phytoseiulus persimilis*. Attraction of *P. persimilis* to the volatiles from nine jasmonic-acid (JA)-induced lima bean leaves, a combination of the volatiles from nine JA-induced lima bean leaves and MeSA (0.2 μg), and the volatiles from four *Tetranychus urticae*-infested lima bean leaves, all tested vs. clean lima bean leaves. Numbers in bars are the absolute numbers of predatory mites responding to each odor source. The triangles represent the percentage of predatory mites in each replicate experiment that was attracted to the presented volatile blends (filled triangles: satiated predatory mites, open triangles: starved predatory mites). Choices between odor sources were analyzed with a one-sided binomial test; the effect of starvation level was compared with a GLM (ns $P > 0.05$; $^{*}P < 0.05$; $^{***}P < 0.001$).

Qualitative Differences in Amount of Methyl Salicylate. The volatiles from JA-induced lima bean leaves attracted satiated *P. persimilis* females (Figure 4, 68%, $P = 0.001$, binomial test), but starved mites were not attracted (55%, $P = 0.22$). A direct statistical comparison of the responses of both groups of mites showed that the effect of starvation level was almost significant ($P = 0.060$, GLM). However, in three out of four replicate experiments, the satiated mites were attracted more to the volatiles from JA-induced lima bean than starved mites.

The satiated mites were strongly attracted to the volatiles from JA-induced lima bean leaves plus 0.2-μg MeSA (Figure 4, 87%, $P < 0.001$, binomial test). The response of the starved mites to the combination of JA-induced volatiles plus synthetic MeSA bordered significance (60%, $P = 0.057$). The behavioral

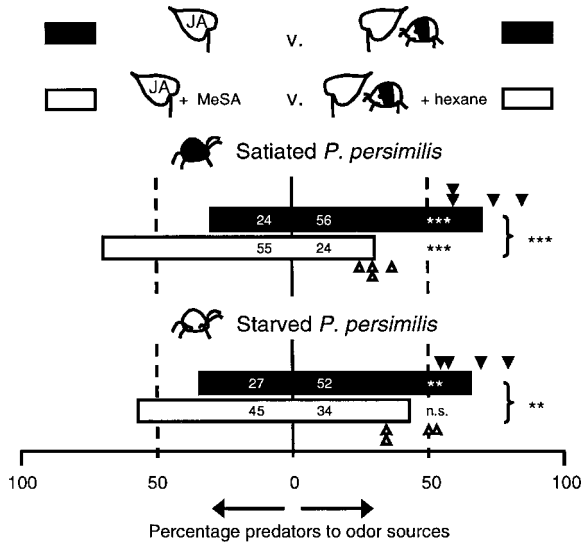

FIG. 5. Choice of satiated and starved *Phytoseiulus persimilis* between the volatiles from *Tetranychus urticae*-induced lima bean leaves and JA-induced lima bean leaves (black bars) and between the volatiles from *T. urticae*-induced lima bean leaves and the combination of JA-induced lima bean leaves plus 0.2-μg MeSA (white bars). Numbers in bars are the absolute numbers of predatory mites responding to each odor source. The triangles represent the percentage of predatory mites in each replicate experiment that was attracted to *T. urticae*-infested lima bean leaves before (filled triangles) and after (open triangles) adding MeSA to the JA-induced lima bean volatiles. Choices between odor sources were analyzed with a two-sided binomial test; the effect of adding MeSA to JA-induced leaves was analyzed with a GLM (ns $P > 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

responses of satiated and starved predatory mites to JA-induced volatiles plus MeSA were different ($P < 0.001$, GLM). In all four replicate experiments, more satiated than starved mites preferred the volatiles from JA-induced lima bean plus MeSA to uninfested lima bean. Both satiated and starved *P. persimilis* females were attracted to the volatiles from *T. urticae*-infested lima bean leaves (Figure 4, $P < 0.001$, binomial test).

Satiated predatory mites preferred *T. urticae*-induced lima bean volatiles to JA-induced volatiles (Figure 5, $P < 0.001$, binomial test). However, when 0.2-μg MeSA was added to the JA-induced volatiles, the preference of *P. persimilis* shifted towards this odor source (from 30 to 70%, $P < 0.001$, GLM). In all four replicates, more mites were attracted to the volatiles from JA-induced lima bean leaves when MeSA was added.

Starved *P. persimilis* females also preferred the volatiles from *T. urticae*-induced lima bean leaves to the volatiles from JA-induced leaves (Figure 5,

$P = 0.007$). Adding MeSA to the JA-induced volatile blend increased the percentage of mites attracted to this odor source in all replicate experiments (from 34% to 57%, $P = 0.004$, GLM). Starved females did not discriminate between the combination of JA-induced volatiles plus MeSA and the *T. urticae*-induced lima bean volatiles ($P = 0.26$, binomial test).

DISCUSSION

In this work, we studied the role of methyl salicylate as part of the volatile blend, in the prey-searching behavior of the predatory mite *P. persimilis*. We showed that the presence of MeSA rather than its relative contribution to a blend of volatiles is important in enabling predators to discriminate between two volatile blends.

Dose-Dependent Attraction to Methyl Salicylate. The single-compound MeSA attracted the predatory mite over a range of doses. The most attractive dose matches roughly with the amount of MeSA emitted by *T. urticae*-infested lima bean plants (estimated from Dicke et al., 1999: 10 *T. urticae*-induced lima bean leaves produced 0.2–0.4 μg MeSA in 30 min). The mites were repelled when they were offered a large, biologically unrealistic, amount of MeSA (200 μg). A low dose of 0.002 μg was not attractive and may have been under the mites, detection level. In an electrophysiological study, De Bruyne et al. (1991) demonstrated a dose-dependent sensitivity of chemoreceptor cells on the anterior tarsi of *P. persimilis* to MeSA, and this could be the basis for a dose-dependent behavioral response to MeSA by the mites.

Quantitative and Qualitative Differences in Volatile Blends. Most plant species that have been investigated so far have shown quantitative differences in volatile blends when they are infested with different herbivore species (e.g., Geervliet et al., 1997; De Moraes et al., 1998; Turlings et al., 1998). Because plants infested with prey or nonprey herbivores are different in profitability for a carnivore, one might hypothesize that quantitative variation is important to a foraging carnivore. However, *P. persimilis* females discriminated between volatile blends that differed qualitatively with respect to the amount of MeSA, but not between blends with a quantitative difference in amount of MeSA.

Both satiated and starved mites preferred *T. urticae*-induced lima bean volatiles to JA-induced volatiles. The absence of MeSA in the JA-induced blend is one of the major differences when compared to the *T. urticae*-induced volatile blend (Dicke et al., 1999). After adding MeSA to the JA-induced blend, the preference of the mites changed significantly. This change indicates an important role for MeSA as a signal to the foraging mites. The amount of MeSA that we added to the set of JA-induced lima bean leaves was of the same order of magnitude

as the amount that the set of *T. urticae*-induced leaves produced (based on Dicke et al., 1999, see above). The preference of satiated predatory mites shifted toward the combination of JA-induced volatiles and MeSA, whereas the response of the starved mites shifted to no discrimination between the two odor sources. This result is in agreement with the stronger preference of satiated compared to starved predatory mites for the combination of JA-induced lima bean leaves plus MeSA when tested against uninfested lima bean leaves.

In contrast, neither satiated nor starved *P. persimilis* females discriminated between *T. urticae*-induced odor blends that differed quantitatively in the amount of MeSA. The response of satiated mites to *T. urticae*-infested leaves was only affected when a large amount of MeSA (200 μg) was added. This repellent amount of MeSA may have masked the presence of the natural volatile blend. The results do not imply that the mites are not able to discriminate among blends with quantitative differences in MeSA or other compounds. They may not always need to discriminate, because not all odor variation represents a different value in terms of the availability of prey or nonprey. Ozawa et al. (2000) showed that *T. urticae*, but not the caterpillar *S. exigua*, induces the production of MeSA in lima bean, i.e., an example of a qualitative difference induced by different herbivore species on the same plant. Alternatively, *P. persimilis* females may need to learn to discriminate among odor sources that differ quantitatively in the amount of MeSA or another compound. The parasitoid *Leptopilina heterotoma* did not discriminate between two odor sources that differed in the quantity of a single component of the blend, unless it had learned to do so (Vet et al., 1998).

Starvation Level and Rearing History. We included both satiated and starved mites in our study because the starvation level may influence the foraging behavior of *P. persimilis* (Dicke et al., 1998; Shimoda and Dicke, 2000). We observed a difference in response of satiated and starved *P. persimilis* females in experiments with JA-induced lima bean volatiles: (1) The volatile blend of JA-induced lima bean attracted satiated, but not starved, predators, (2) Satiated mites were significantly more attracted to a combination of JA-induced lima bean volatiles plus MeSA than starved mites, (3) Satiated mites preferred a combination of JA-induced lima bean volatiles plus MeSA to *T. urticae*-induced lima bean volatiles, whereas starved predators did not discriminate between these odor sources. These data lend support to the hypothesis that starved predatory mites are more selective than satiated mites in response to volatiles (Shimoda and Dicke, 2000). These authors suggested that it would be adaptive for satiated mites to respond to incomplete volatile information, i.e., only a part of the volatile blend that is produced by prey-infested plants. For example, they could use such a subset of volatiles when they return to a prey patch that they left but that is still nearby (Sabelis et al., 1984; Shimoda and Dicke, 2000). Surprisingly, both groups of mites were attracted to MeSA—also an incomplete

volatile blend in itself—supporting an important role for this compound in the foraging behavior of *P. persimilis*.

Several studies have demonstrated that the rearing history of *P. persimilis* influences its subsequent choices (Takabayashi and Dicke, 1992; Krips et al., 1999; Shimoda and Dicke, 2000). In our study, the predatory mites were reared on spider mites on lima bean. The predators were, thus, exposed to MeSA during the rearing (Dicke et al., 1990). This probably explains their attraction to the single-compound MeSA. The effects of rearing history and learning on the predatory mites' response to MeSA need to be investigated.

In summary, this study is a new step in unraveling which parts of the complex herbivore-induced volatile blends contain information that carnivores use to locate their prey. Using the combined knowledge of the composition of two volatile blends and the differential responses of a carnivore, we studied the role of MeSA in the foraging behavior of the predatory mite *P. persimilis*. We conclude that MeSA can be a signal that determines the predator's preference in a choice situation between two volatile blends that differ in the presence of MeSA. However, MeSA can clearly not be the only compound used by *P. persimilis* to identify the presence of spider mites. The common presence of MeSA in herbivore-induced volatile blends, and the absence of MeSA in the spider mite-induced volatile blends of some plant species, rule out a role for MeSA as the single compound that reveals the presence of spider mites on a plant (e.g., Scutareanu et al., 1997; Krips et al., 1999; Turlings et al., 1998; Van den Boom et al., in press). Other herbivore-induced volatiles are known to attract satiated *P. persimilis* females as a single compound: linalool, β-ocimene, and 4,8-dimethyl-1,3(*E*),7-nonatriene (Dicke et al., 1990). The role of these compounds as part of a volatile blend has not yet been investigated. They may be important when MeSA is not present or in combination with MeSA. By using chemical analytical methods, electroantennograph studies, and behavioral assays, it will be possible to make a directed search for compounds that may contain relevant information for foraging carnivores.

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THE ROLE OF HONEYDEW IN HOST SEARCHING OF APHID HYPERPARASITOIDS

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Abstract—Foraging in many insect parasitoids is mediated by chemicals associated with hosts. For example, honeydew, the feces of feeding aphids, induces and/or prolongs searching behavior of aphid parasitoids. In the laboratory, we tested if aphid hyperparasitoids, which belong to a higher trophic level, also rely on aphid honeydew to locate their hosts. We used the potato aphid, *Macrosiphum euphorbiae*, the primary parasitoid, *Aphidius nigripes*, and four hyperparasitoids, *Asaphes suspensus*, *Dendrocerus carpenteri*, *Alloxysta victrix*, and *Syrphophagus aphidivorus* that possess different biological attributes and host ranges. We determined if foraging hyperparasitoid females could discriminate between (i) honeydew from a host and a non-aphid host (the potato aphid and the soft brown scale, *Coccus hesperidum*), and (ii) honeydew from healthy aphids and those parasitized by *A. nigripes*. Females of *A. suspensus* did not react to any of the honeydew treatments. While the presence of non-aphid honeydew did not modify the behavior of *A. victrix*, *D. carpenteri*, and *S. aphidivorus* females, they exhibited an increase in searching time and path length but not walking speed when in the presence of honeydew from aphids. However, there were no changes in host searching behaviors, such as antennation or ovipositor probing that have been reported for primary aphid parasitoids. There was no difference in the response of hyperparasitoid females to honeydew from healthy and parasitized aphids. These results indicate that hyperparasitoids may use aphid honeydew,

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a conspicuous cue from the second trophic level, as an infochemical to locate their hosts.

Key Words—Honeydew, aphid, aphid parasitoid, hyperparasitoid, host searching behavior, trophic interactions, infochemical detour.

INTRODUCTION

Honeydew, a complex mixture of chemical compounds, of which the most important are sugars and amino acids (Auclair, 1963) is excreted by phloem-feeding Homoptera, such as aphids, whiteflies, and scale insects. Differences in chemical composition of aphid honeydew have been studied in detail and may vary depending on the host plant species (Hendrix et al., 1992; Douglas, 1993; Fisher and Shingleton, 2001), the aphid species (Hendrix et al., 1992; Völkl et al., 1999; Fisher and Shingleton, 2001), the aphid age (Fisher et al., 2002), the sugar concentration in the diet (Mittler and Meikle, 1991; Wilkinson et al., 1997), the level of ant tending (Fisher and Shingleton, 2001; Yao and Akimoto, 2001), the presence of bacterial intracellular symbionts (Sasaki et al., 1990; Wilkinson and Douglas, 1995; Wilkinson et al., 1997), and parasitism (Cloutier, 1986). Honeydew may serve as a source of carbohydrates for many insects, e.g., ants and parasitoids (Völkl et al., 1999; Wäckers and Steppuhn, 2003).

Honeydew is also used as an infochemical by foraging parasitoids (e.g., Bouchard and Cloutier, 1984) and predators (e.g., Budenberg and Powell, 1992). Its role in host searching of aphid parasitoid females has been studied extensively. Honeydew attracts foraging parasitoid females (Bouchard and Cloutier, 1985; Wickremasinghe and van Emden, 1992) and/or arrests them on contaminated areas (Bouchard and Cloutier, 1984; Gardner and Dixon, 1985; Ayal, 1987; Budenberg, 1990; Cloutier and Bauduin, 1990; Hågvar and Hofsvang, 1991; Budenberg et al., 1992; Grasswitz and Paine, 1993). Honeydew may also contain substantial specific information for natural enemies, for while *Aphidius rhopalosiphi* females respond to honeydew of both host and nonhost aphids, they spend less time in areas contaminated with honeydew from the nonhost species (Budenberg, 1990).

Aphid parasitoids can in turn be parasitized by different species of hyperparasitoids. Contrary to primary parasitoids, honeydew from healthy aphids does not appear to attract hyperparasitoids towards contaminated areas (Buitenhuis et al., unpublished). This is not altogether surprising, for while honeydew is a direct cue to the presence of aphids for parasitoids, it would only be an indirect cue for hyperparasitoids, as it provides females no reliable information about the presence of their aphid parasitoid hosts. On the other hand, honeydew does act as a contact synomone, inducing hyperparasitoid females to stay and search longer on contaminated surfaces and plants (Budenberg, 1990; Grasswitz, 1998; Buitenhuis et al., unpublished). However, parasitism by braconid wasps may also induce changes

in both the quantity and composition of honeydew produced by aphids (Cloutier and Mackauer, 1979; Cloutier, 1986; Rahbé et al., 2002). Therefore, honeydew could be a direct and reliable cue for hyperparasitoids if females have evolved the capacity to discriminate between honeydew from healthy and parasitized aphids.

In this study, we examined the innate response of aphid hyperparasitoids to different types of honeydew. We predicted that foraging hyperparasitoid females not only have the ability to detect honeydew, but also show a preference for honeydew from aphid rather than non-aphid species and, more specifically, for honeydew from parasitized aphids. We tested these predictions in the laboratory by measuring behavioral components of hyperparasitoid females exposed to water extract of honeydew applied to filter paper discs following the study of Bouchard and Cloutier (1984). We used the potato aphid, *Macrosiphum euphorbiae* (Thomas), its primary parasitoid, *Aphidius nigripe*s Ashmead, and four hyperparasitoids, *Asaphes suspensu*s Walker (Pteromalidae), *Dendrocerus carpenteri* (Curtis) (Megaspilidae), *Alloxysta victrix* (Westwood) (Alloxystidae), and *Syrphophagus aphidivorus* (Mayr) (Encyrtidae). These species were chosen because while they all naturally exploit *Aphidius* spp., they possess different biological attributes and host ranges. *A. suspensus* and *D. carpenteri* are generalist ectoparasitoids that attack primary parasitoid pupae following mummification of the aphid. *A. victrix* is an endoparasitoid that lays its egg in parasitoid larvae prior to aphid mummification and has a more restricted host range. Finally, *S. aphidivorus* is a generalist hyperparasitoid with the capacity to attack either primary parasitoid larvae in live aphids or parasitoid pupae following mummification.

METHODS AND MATERIALS

Insects. Colonies of the aphid, parasitoid, and four hyperparasitoids were reared on potato seedlings following the techniques of Brodeur and McNeil (1994) and Buitenhuis et al. (unpublished). To prevent contact with honeydew stimuli before the test, hyperparasitized aphid mummies were collected, put in individual gelatine capsules, and kept at 20±1°C, 75±10% RH, under a 16L:8D photoperiod until adult emergence. Adults were sexed, and females were put into small individual ventilated cylindrical cages (5 cm diam and 10 cm high) with a supply of 40% sucrose solution and held under the same conditions until used.

Honeydew Collection. Honeydew was collected by placing Parafilm sheets for 24 hr under potato plants infested with either healthy aphids from all developmental stages or parasitized aphids. Parasitized aphids were obtained by exposing third instars for 24 hr to 3–5 day-old mated females at a parasitoid: host ratio of 1:2 (resulting in 90–95% parasitism). Honeydew was collected 4–7 days later. The response of hyperparasitoid females to honeydew from a nonhost was tested by using honeydew from scale insects (*Coccus hesperidum* L.: Coccidae) collected on *Ficus benjamina* L. (Moraceae) plants. In this instance, both herbivore and plant

species were different from the potato-aphid system. This honeydew was collected in the same manner as described above but, due to the lower insect density, the Parafilm sheets were removed after 2 days. Honeydew was allowed to dry for 30 min at 40°C, collected by scraping the sheets with a glass microscope slide and then stored at −20°C until use. Before the bioassay, honeydew was weighed and dissolved in distilled water, filtered through a cloth, and adjusted to a concentration of 0.26 mg/μl (following Bouchard and Cloutier, 1984). Between bioassays, the solution was stored at 4°C for a maximum of 8 days.

Bioassay. One hundred μl of the distilled water, honeydew from either healthy or parasitized aphids, or from nonhost scales were applied in the middle of a filter paper (12.5 cm diam; Schleicher & Schuell #595), giving a treated circle about 4 cm diam. A circle of 12 cm diam was drawn inside the perimeter of the disc. The paper was dried and used within 5 hr of preparation. For each assay, the test paper was placed into a 14 cm diam glass petri dish, covered by a glass plate, and located in a tent lit by a circular 22 W fluorescent tube. One virgin, naïve, female hyperparasitoid (2–7 day old) was released onto the middle of the filter paper and her behavior recorded on video until she either crossed the 12 cm circle or until she flew to the side or top of the arena. Females that immediately flew off the filter paper or that did not move were excluded from the analysis. One female of each of the four hyperparasitoid species was tested on the same filter paper. Filter papers treated with aphid honeydew were used only once, but in the case of scale honeydew assays, they were used twice due to the shortage of scale honeydew solution. Such a procedure had no effect on any of the measured parameters: residence time, path length, or walking speed (2-way ANOVAs using hyperparasitoid species and 1st/2nd repetition as factors, all P-values >0.05). In all assays, the hyperparasitoid species were randomized within treatments, and 20 replicates per treatment were done within an 8 day period. The time spent inside and outside the contaminated area was determined from the videotape using the Observer (Noldus, information technology, version 3 for Macintosh), while the locomotory behavior was quantified by tracing each female's path on a transparency and then measuring its length. Walking speed (cm/sec) was calculated by dividing the total path length by the total time.

Statistical Analysis. Duration and path length data were $\log(x + 1)$ transformed, whereas speed data were square root transformed prior to being analyzed with a two-way ANOVA. Differences between treatments were determined by contrasts. Given that statistical models had three degrees of freedom per factor, only three orthogonal contrasts ($\alpha = 0.05$) were allowed. To test the predictions, we selected *a priori* (i) honeydew, regardless of origin vs. water, (ii) aphid vs. non-aphid (scale) honeydew, and (iii) honeydew from healthy and parasitized aphids. One additional contrast analysis, (iv) non-aphid (scale) honeydew vs. water, was done by using Scheffé's adjustment of the P-value (Steel and Torrie, 1980). Differences between hyperparasitoid species were determined by Fisher's

protected LSD ($\alpha = 0.05$). All analyses were done using SAS (SAS Institute, 1999).

RESULTS

Most females of all hyperparasitoid species walked on the filter paper: *A. victrix* (77%), *A. suspensus* (88%), *D. carpenteri* (94%), and *S. aphidivorus* (100%). In all species, walking was continuous or interrupted with short jumps. The trajectories of females that did or did not respond to honeydew were different. Females in the control treatment, and those not responding to honeydew, usually walked rapidly across the treated area without showing evidence of arrestment (Figure 1). In contrast, a positive response was characterized by a klinotactic response, and the resulting tortuous path ensured that the females searched most of the treated area (Figure 1).

Overall, there were significant effects of both treatment and species on residence times and path length (Table 1, Figures 2 and 3). However, while there were species specific differences in walking speed for any given species, this parameter was unaffected by treatment (Table 1, Figure 4). These treatment effects are due to overall differences in response to aphid honeydew compared with those to water and honeydew from scale insects (Table 2, Figures 2 and 3). However, the contrast analyses underlined specific treatment differences among the four hyperparasitoid species (Table 2). One notable point is that *A. suspensus* female foraging behaviors remained unchanged in all assays (Table 2, Figures 2 and 3). The apparent increased time spent in the scale insect treatment (Figure 2) was nonsignificant and

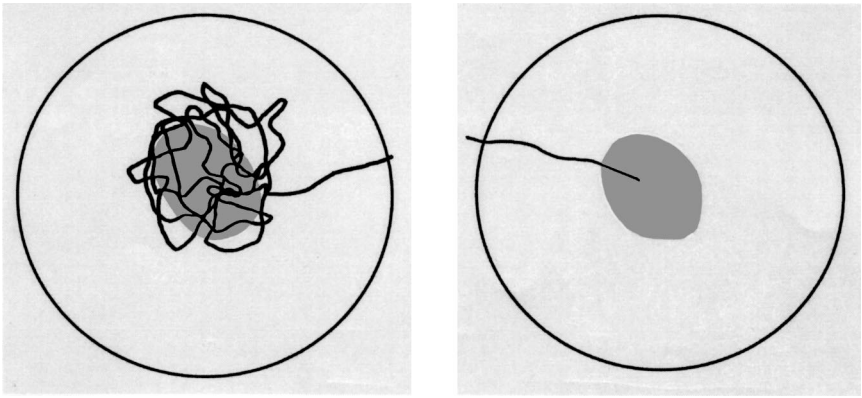

FIG. 1. Typical path tracings of aphid hyperparasitoid females that responded (left, *Dendrocerus carpenteri* on honeydew from parasitized aphid) or not (right, *D. carpenteri* on honeydew from scale insect) to honeydew.

TABLE 1. RESULTS OF 2-WAY ANOVAS ON DIFFERENT PARAMETERS DESCRIBING THE BEHAVIOR OF FOUR SPECIES OF APHID HYPERPARASITOIDS FORAGING ON A FILTER PAPER DISC TREATED WITH DIFFERENT HONEYDEW EXTRACTS

	Factors		
Parameters	Honeydew treatment	Hyperparasitoid species	Interaction
Total residence time	$F_{3,252} = 11.78$, $P < 0.001$	$F_{3,252} = 3.43$, $P = 0.018$	$F_{9,252} = 1.40$, $P = 0.186$
In treated patch	$F_{3,252} = 8.62$, $P < 0.001$	$F_{3,252} = 4.91$, $P = 0.002$	$F_{9,252} = 1.79$, $P = 0.071$
Outside treated patch	$F_{3,252} = 9.16$, $P < 0.001$	$F_{3,252} = 3.63$, $P = 0.014$	$F_{9,252} = 0.56$, $P = 0.827$
Path length	$F_{3,252} = 16.62$, $P < 0.001$	$F_{3,252} = 26.78$, $P < 0.001$	$F_{9,252} = 1.03$, $P = 0.413$
Walking speed	$F_{3,252} = 1.53$, $P = 0.207$	$F_{3,252} = 54.42$, $P < 0.001$	$F_{9,252} = 0.51$, $P = 0.868$

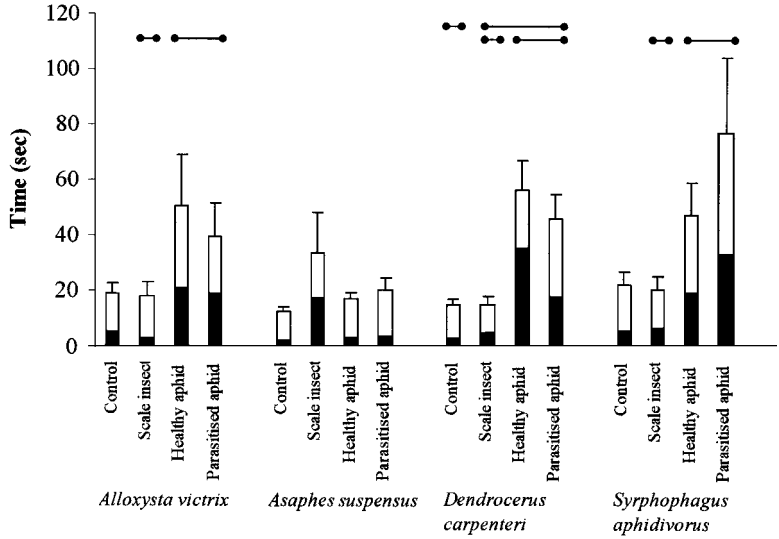

FIG. 2. Residence time (mean + SE) of female of four species of aphid hyperparasitoids foraging on a filter paper disc (12 cm diam) treated with different honeydew extracts. Bars indicate time spent within (black) and outside (white) treated area. Per species, significant contrasts for total residence time spent on the filter paper are indicated with horizontal bars. For details on all statistical differences, see Table 2.

resulted from the behavior of two females, one which spent a long time walking in the patch and the other that remained outside the patch for a prolonged period. When the pooled responses to honeydew, regardless of source, and water were compared, *D. carpenteri* females showed significant changes in foraging, while *A. victrix* and *S. aphidivorus* did not. However, there were no differences between water and scale honeydew for any given species (Table 2) while all responded to aphid honeydew. Contrary to our initial hypothesis, there were no differences between honeydew from healthy and parasitized aphids (Table 2, Figures 2 and 3).

DISCUSSION

These results, together with those of Budenberg (1990) on *Alloxysta macrophadna* and *Phaenoglyphis villosa* (Alloxystidae), and Grasswitz (1998) on *A. victrix*, indicate that aphid honeydew may modify female foraging behavior in species from each of the three subfamilies (Cynipoidea, Ceraphronoidea, Chalcidoidea) where aphid hyperparasitoids are found. The existence of such a common response among evolutionairly diverse groups of aphid hyperparasitoids suggests that aphid

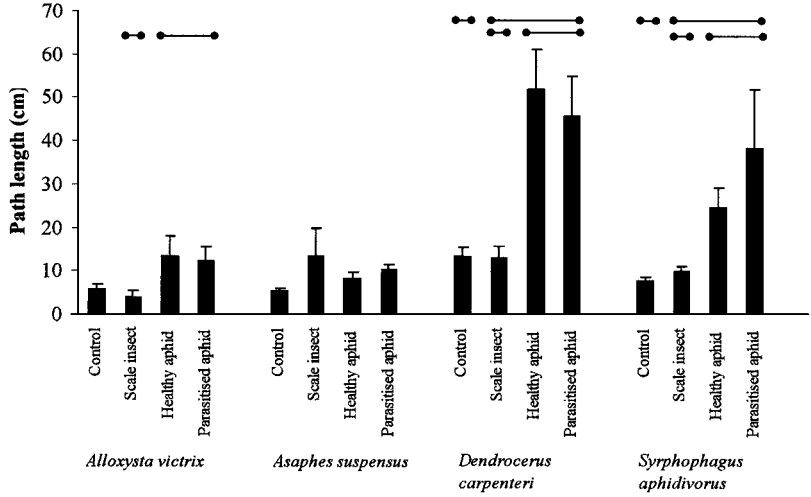

FIG. 3. Path length (mean + SE) of female of four species of aphid hyperparasitoids foraging on a filter paper disc (12 cm diam) treated with different honeydew extracts. Per species, significant contrasts are indicated with horizontal bars. For details on all statistical differences, see Table 2.

honeydew is a reliable cue to host finding and may serve as a contact synomone that transcends trophic levels. A parallel study, on a different spatial scale that used whole plants, also showed that the foraging behavior of hyperparasitoid females was modified by the presence of aphid honeydew (Buitenhuis et al., unpublished). This conclusion is supported by the fact that the behavioral changes observed were not in response to all sources of honeydew, but rather to honeydew produced by insects serving as a host for the primary parasitoid. This ability to discriminate between aphid and non-aphid honeydew results in females making extensive searches in areas where aphid parasitoids are most likely to be found. Honeydew composition is in a large part determined by the elements of phloem sap, and is, thus, plant specific (Hendrix et al., 1992), so we cannot exclude the possibility that the different patterns observed may be associated with different host plants, i.e., potato vs. *Ficus* plants used by the two herbivore species. However, discrimination between host and nonhost honeydew has been reported, as the whitefly parasitoid *Encarsia formosa* responded differently to whitefly and aphid honeydew, when both species were reared on the same host plant (Romeis and Zebitz, 1997).

Not all hyperparasitoids respond in the same way to honeydew from hosts exploited by primary parasitoids. While three of the four species modified their behavior, one, *A. suspensus*, did not. Furthermore, no consistent patterns of response to aphid honeydew are found when considering aphid hyperparasitoids with different

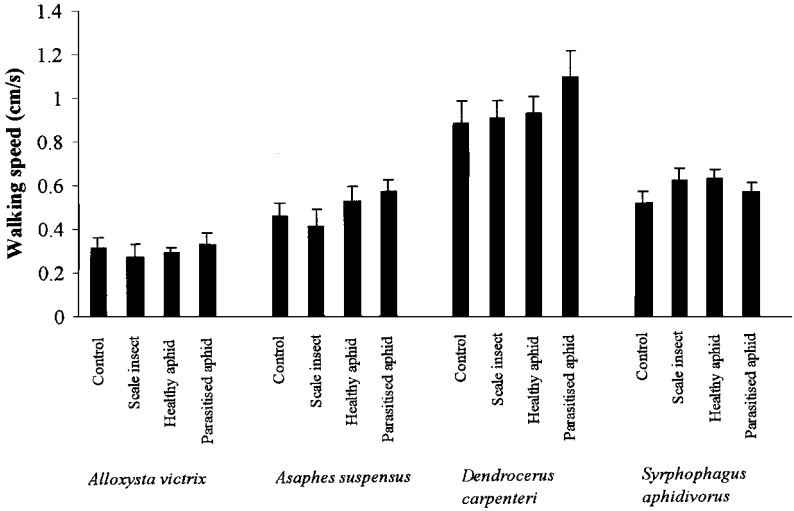

FIG. 4. Walking speed (mean + SE) of female of four species of aphid hyperparasitoids foraging on a filter paper disc (12 cm diam) treated with different honeydew extracts. No species showed significant differences between treatments.

life-history strategies (endo- vs. ectoparasitoids, koino- vs. idiobiont parasitoids) and the type of primary parasitoid host attacked (parasitoid larva in live aphid or parasitoid pupa in aphid mummy). Similarly, host specificity does not appear to shape aphid hyperparasitoid responses to honeydew. For example, *A. victrix*, a koinobiont hyperparasitoid, has a narrower host spectrum than most idiobiont hyperparasitoids, including those tested in this study (Brodeur, 2000), but showed the same type of response. In contrast, the foraging of *A. suspensus*, a cosmopolitan and polyphagous species (Höller, et al., 1993), was unaffected by honeydew on the substrate (this study) or by aphids/honeydew on plants (Buitenhuis, et al., unpublished). Nevertheless, it is possible that the use of honeydew as a foraging cue can be learned. More species must be examined in order to explain such marked differences in preference or absence of response.

Despite the potential advantages of recognizing honeydew from parasitized aphids, females of the hyperparasitoid species we tested did not discriminate between honeydew from healthy and parasitized aphids. Several nonexclusive explanations may account for this. First, differences between honeydew from healthy and parasitized aphids are mostly reflected in quantitative differences in amino acids (Cloutier, 1986). Furthermore, while the presence of primary parasitoid larvae may modify aphid honeydew, several other factors may result in similar changes. These include aphid and host plant species, which may modify the nature and concentration of amino acids and sugars present (Douglas, 1993; Völkl et al., 1999; Fisher

TABLE 2. *P*-VALUES OF CONTRAST ANALYSES ON THE EFFECT OF DIFFERENT TYPES OF HONEYDEW ON THE BEHAVIOR OF FOUR SPECIES OF APHID HYPERPARASITOIDS

		Visit time				
Species	Contrast	Inside honeydew	Outside honeydew	Total	Path length	Walking speed
Alloxysta. victrix	Control vs. all honeydew	0.386	0.133	0.187	0.252	0.879
	Scale insect vs. aphid	0.018*	0.080	0.020*	0.009*	0.569
	Healthy aphid vs. parasitized aphid	0.844	0.685	0.762	0.844	0.840
	Control vs. scale insect[a]	0.410	0.988	0.641	0.437	0.650
Asaphes suspensus	Control vs. all honeydew	0.127	0.240	0.177	0.130	0.571
	Scale insect vs. aphid	0.178	0.494	0.722	0.418	0.090
	Healthy aphid vs. parasitized aphid	0.656	0.943	0.774	0.409	0.544
	Control vs. scale insect[a]	0.052	0.639	0.223	0.517	0.554
Dendrocerus carpenteri	Control vs. all honeydew	0.004*	0.308	0.008*	0.002*	0.310
	Scale insect vs. aphid	0.002*	0.001*	<0.001*	<0.001*	0.547
	Healthy aphid vs. parasitized aphid	0.098	0.595	0.386	0.452	0.389
	Control vs. scale insect[a]	0.770	0.244	0.796	0.983	0.687
Syrphophagus aphidivorus	Control vs. all honeydew	0.092	0.128	0.057	0.003*	0.125
	Scale insect vs. aphid	0.038*	0.004*	0.003*	0.004*	0.873
	Healthy aphid vs. parasitized aphid	0.736	0.234	0.348	0.628	0.478
	Control vs. scale insect[a]	0.948	0.571	0.755	0.558	0.204

Note. Contrast treatments were: control (distilled water); all honeydew (combination of all honeydew treatments); aphid (honeydew produced by *Macrosiphum euphorbiae*, both healthy and parasitized by *Aphidius nigripes*); scale insect (honeydew produced by *Coccus hesperidum*); healthy aphid (honeydew produced by *M. euphorbiae*); parasitized aphid (honeydew produced by *M. euphorbiae* parasitized by *A. nigripes)*. Significant contrasts are indicated by asterisks ($P < 0.05$).

[a] Additional contrast with Scheffé adjustment.

and Shingleton, 2001). In addition, hyperparasitoid females foraging in an aphid colony under natural conditions will encounter a mix of new and decomposing honeydews from both healthy and parasitized aphids, which could mask any subtle quantitative differences associated with the origin of the synomone. One must conclude that differences between honeydew from healthy and parasitized aphids do not provide sufficiently reliable cues to modify foraging behavior.

Primary parasitoids and hyperparasitoids of aphids both use aphid honeydew in host searching, and the response to this cue appears to be innate, as naïve females respond to the infochemical (Bouchard and Cloutier, 1984; Grasswitz and Paine, 1993; Grasswitz, 1998). There are, however, distinct differences between the two trophic levels. While both use honeydew as an arrestant cue, primary parasitoids use volatiles from honeydew in long distance search (Bouchard and Cloutier, 1985), while hyperparasitoids do not (Buitenhuis et al., unpublished). Furthermore, when primary parasitoids contact host honeydew, there are a series of behavioral changes, including increased antennation, abdominal extension/flexing, reduced walking speed, and increased turning rate (Bouchard and Cloutier, 1984; Budenberg, 1990; Hågvar and Hofsvang, 1991). Female hyperparasitoids also spend longer times and follow tortuous paths when encountering honeydew patches, yet they maintain a constant walking speed and do not perform the specific behaviors seen in primary parasitoids. Such differences may arise from differences in the reliability of aphid honeydew as a foraging cue for primary and secondary parasitoids. Honeydew represents a reliable, abundant, and direct source of information about the presence of hosts to primary parasitoids (Vet and Dicke, 1992). In contrast, it provides no reliable information about the availability of suitable stages of the primary parasitoid that the hyperparasitoid females exploit. This situation represents an example of an infochemical detour, where the cue is only indirectly related to its host/prey (Vet and Dicke, 1992). Aphid hyperparasitoid females could benefit from searching in habitats contaminated by honeydew, as parasitized aphids and aphid mummies can be found either within or near the aphid colony (Brodeur and McNeil, 1989, 1992; Müller et al., 1997). Furthermore, by keeping a constant walking speed, females possibly cover a greater area and, thus, gain the greatest benefit from an indirect cue for host availability.

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HYDROXYBENZOIC ACID DERIVATIVES IN A NONHOST RUTACEOUS PLANT, *Orixa japonica*, DETER BOTH OVIPOSITION AND LARVAL FEEDING IN A RUTACEAE-FEEDING SWALLOWTAIL BUTTERFLY, *Papilio xuthus* L.

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Abstract—A Rutaceae-feeding swallowtail butterfly, *Papilio xuthus* L., feeds on various rutaceous plants but always rejects *Orixa japonica* Thunb. (Rutaceae). Females were strongly deterred from laying eggs by a methanolic extract of *O. japonica* leaves. Larvae also rejected a diet leaf medium impregnated with *O. japonica* leaf extracts. Several components in the water-soluble fraction of the leaf extract were found to deter both oviposition and feeding responses. Two major deterrent compounds were characterized as 5-{[2-*O*-(*β*-D-apiofuranosyl)-*β*-D-glucopyranosyl]oxy}-2-hydroxybenzoic acid and a disyringoyl aldaric acid. These compounds induced potent deterrence of both oviposition and larval feeding by *P. xuthus*, which suggests a congruent chemosensory mechanism of allomonal chemicals acting on both female tarsal chemoreceptors and larval maxillary taste receptors.

Key Words—*Papilio xuthus*, *Orixa japonica*, Rutaceae, oviposition deterrent, feeding deterrent, 5-{[2-*O*-(*β*-D-apiofuranosyl)-*β*-D-glucopyranosyl]oxy}-2-hydroxybenzoic acid, aldaric acid disyringoyl ester, host selection.

INTRODUCTION

A large number of swallowtail butterfly species in the genus *Papilio* utilize host plants in the family Rutaceae. Among them, *Papilio xuthus* L. uses various *Citrus* plants and certain species in other rutaceous genera, such as *Poncirus*, *Zanthoxylum*, *Fagara*, and *Phellodendron* (Fukuda et al., 1982). However, *Orixa*

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japonica Thunb. also in the Rutaceae is never accepted by *P. xuthus* even through the plant is a major host of some related species, such as *P. bianor* and *P. macilentus* (Abe et al., 1981; Nishida, 1995). *P. xuthus* females do not lay eggs, and the larvae do not feed on the foliage of *O. japonica*. Host-plant chemistry plays a major role in host acceptance or rejection by papilionid butterflies (Honda and Hayashi, 1995a; Nishida, 1995). At oviposition, female butterflies perceive the phytochemical information through their tarsal sensilla by drumming upon the leaf surface (Feeny et al., 1983; Nishida, 1995). A qualitative and quantitative balance of stimulants and deterrents governs acceptance or rejection of the plant (Honda and Hayashi, 1995a). As for larval feeding, plant chemicals on the leaf surface and/or released from the leaf interior upon biting seem to strongly influence the decision whether or not feeding will be continued. Host choice is, thus, controlled by plant allelochemicals that are perceived by the female's tarsal chemoreceptors and the larval gustatory chemoreceptors (Schoonhoven et al., 1998).

The relationship between oviposition preference and offspring performance is a central issue in the evolution of host adaptation in swallowtail butterflies (Wiklund, 1975; Thompson, 1988). Because of their limited foraging ability, the larvae are crucially dependent upon the oviposition preferences of their mothers. To the extent that the chemosensory mechanisms of adult and larval host recognition are intrinsically coordinated, females can usually select the correct host plants for the larvae. However, the nature of such coordination between adult and larval chemosensory responsiveness to allellochemicals during host assessment is unclear.

In a previous paper (Nishida et al., 1990), a flavonol triglycoside was identified as one of the oviposition deterrents for *P. xuthus* in leaves of *O. japonica*. Further investigation revealed the presence of deterrent compounds that act on both oviposition and larval feeding. We describe here the isolation and characterization of these allomonal factors that are crucial to the host preference of *P. xuthus*.

METHODS AND MATERIALS

Insects. Eggs of *P. xuthus* were obtained from adults collected from the campus of Kyoto University, Japan. Larvae were raised on leaves of various *Citrus* plants. Insects were kept at $24 \pm 2^{\circ}$C under a photoperiod of 16L/8Days for rearing and bioassay. Females were hand-paired within 2 d of emergence and fed daily with a honey solution. Gravid females, 3–10 days after hand-pairing, were employed for oviposition bioassay during the daytime. Fifth instars, 3–5 days after molting, were starved for 6–10 hr and then used for feeding bioassay.

Oviposition Deterrent Bioassay. The behavioral bioassay was conducted following the method described in Nishida et al. (1990). Test samples of given doses [gram leaf equivalent per filter paper (g.l.e./f.p.)] were applied to a piece of filter

paper (10 cm^2, fan-shaped). Each female butterfly was placed in turn onto the filter paper and kept in contact for 30 sec. Females responded immediately to filter paper treated with the crude aqueous fraction (postethyl acetate extract) of *Citrus unshiu* S. Marcov. at a dose of 0.03 g.l.e./f.p., curling their abdomens and eventually laying eggs. However, females did not show any oviposition response to a filter paper treated with a mixture of the aqueous extract of *C. unshiu* and a methanolic extract of *O. japonica*. Therefore, oviposition deterrent activity was determined by adding each *Orixa*-fraction (0.0003–0.3 g.l.e./f.p.) to the standard aqueous fraction of *C. unshiu* (0.03 g.l.e./f.p.). Any females either giving a positive response to a moistened blank filter paper or giving no response to a filter paper containing the standard aqueous fraction of *C. unshiu* were rejected from the test. Thus, oviposition deterrent activity was evaluated as the percentage of females rejecting the test samples. Differences in responses between test samples were compared by a χ^2 test.

Feeding Deterrent Bioassay. When larvae were given leaves of the host plant (*Citrus unshiu*), they first palpated with the tips of their maxillary palps, bit, and then swallowed the leaves. Given the leaves of *O. japonica*, they also touched the surface of the plant with the palps, but showed only some exploratory bites without ingesting the leaves. Similar rejection was observed when the host leaves were treated with the *O. japonica* extract. A bioassay to determine the compounds responsible for deterrence of *O. japonica* was devised in light of the following further observations: (1) the larvae did not show feeding behavior to the residue of leaves of *O. japonica* after thorough methanolic extraction; (2) they were stimulated to feed on the residue impregnated with the aqueous fraction of *C. unshiu* that contained feeding stimulants (Figure 1); (3) if the above *Citrus*-extract-treated *Orixa* leaf residue was treated then with *O. japonica* extract, they rejected the leaf material.

For the deterrent bioassay, therefore, the test substrates (leaflets) were prepared by the following method: Fully extended young leaves of *O. japonica* were extracted twice with methanol to remove soluble materials. The residue of each leaf was pressed between sheets of blotting paper to make the "bleached" leaf material flat and dry, after which it was cut into a rectangular shape (2×0.5 cm). Leaflets were soaked in the aqueous fraction of *C. unshiu* (5 g.l.e./ml in water) for more than 12 hr at 4°C and dried under ambient conditions (control leaflets). Test samples of given doses of *O. japonica* extracts [measured as gram leaf equivalents (g.l.e.)] in 5–20-μl aqueous, or methanol solutions were applied to test leaflets (1 cm^2 t.l.) using a micro syringe and dried again. The starved larvae consumed control leaflets but rejected leaflets treated with *Orixa* extracts (no ingestion). Larvae giving no response to the control leaflet were rejected from the test. Feeding deterrent activity was evaluated as the percentage of larvae giving a deterrent response to the test leaflets during a 30-sec test period. Differences in responses between test samples were compared by a χ^2 test.

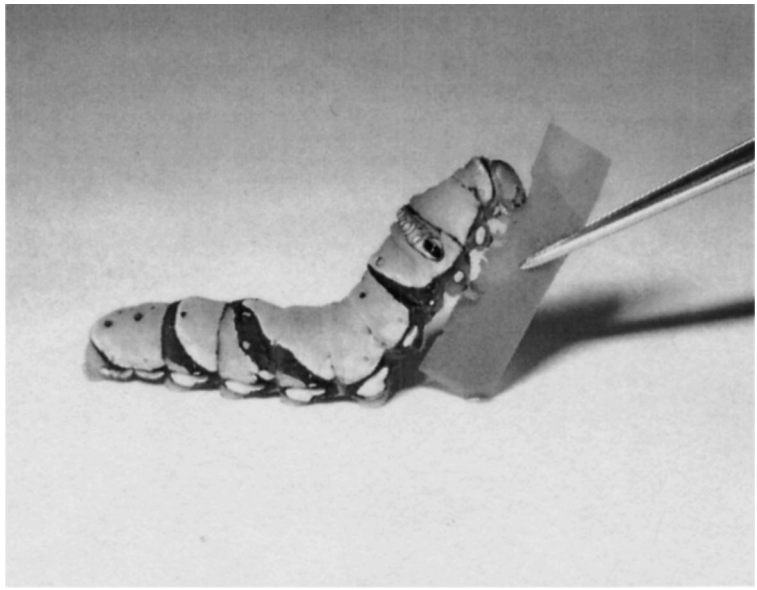


FIG. 1. Feeding deterrent bioassay on a *Papilio xuthus* larva. The larva is positively consuming a leaflet of *Orixa japonica*, which have been "bleached" and then impregnated with an aqueous fraction of *Citrus unshiu* leaves. However, the larva rejected the leaflet if an *O. japonica* extract was added.

Extraction and Fractionation. Fresh leaves of *O. japonica* (4.8 kg) were collected from the campus of Kyoto University in June 1996 and extracted with methanol (18 l × 2). The extract (398 g) was dissolved in water and partitioned with ethyl acetate (ethyl acetate layer, 140 g; water layer, 258 g). A portion of the water layer (27.4 g, 500 g.l.e.) was chromatographed on a reverse-phase column (300 g of Cosmosil 140C18-OPN, 140 μm, Nacalai Tesque, Inc., 380 × 50 mm i.d.), eluted with 1% acetic acid (aq.) (Fr. 1), 10% methanol + 1% acetic acid (aq.) (Fr. 2), 20% methanol + 1% acetic acid (aq.) (Fr. 3), 40% methanol + 1% acetic acid (aq.) (Fr. 4), 60% methanol + 1% acetic acid (aq.) (Fr. 5), 80% methanol + 1% acetic acid (aq.) (Fr. 6), and 100% methanol (Fr. 7) (yield: Fr. 1, 20.3 g; Fr. 2, 1.03 g; Fr. 3, 1.50 g; Fr. 4, 2.29 g; Fr. 5, 1.27 g; Fr. 6, 153 mg; Fr. 7, 100 mg).

*HPLC Separation of Compound **1**.* Fr. 2 (840 mg, 450 g.l.e., after removal of an insoluble solid mass) was chromatographed on a reverse phase HPLC column (YMC-Pack Pro C18, AS-343, 250 × 20 mm i.d., YMC Ltd.) eluted with 30% methanol in 1% acetic acid (aq.) at a flow rate of 5.0 ml/min, and the activity was recovered in a range of $R_t = 27.2$–29.6 min. The eluate was rechromatographed on another reverse phase HPLC column (YMC-Pack ODS-AQ323, 250 × 10 mm i.d.) eluted with 30% methanol in 1% acetic acid (aq.) at a flow rate of 2.0 ml/min.

Crude compound **1** was obtained from $R_t = 12.3$ min, which was further purified on the same column with 12% acetonitrile in 1% acetic acid (aq.) at a flow rate of 2.0 ml/min, and pure **1** was isolated at $R_t = 12.8$ min.

*HPLC Separation of Compound **2**.* Fr. 3 (4.50 g, 1500 g.l.e.) was chromatographed on a reverse phase HPLC column (YMC-Pack *Pro* C18 AS343, 250 × 20 mm i.d., YMC Ltd.) eluted with 30% methanol in 1% acetic acid (aq.) at a flow rate of 7.5 ml/min, and an eluate was collected in a range of $R_t = 25.5$–27.8 min. The eluate was chromatographed further on YMC-Pack ODS-AQ323 (250 × 10 mm i.d., YMC Ltd.) with 12.5% acetonitrile in 1% acetic acid (aq.) at a flow rate of 2.0 ml/min, and an eluate was collected at around $R_t = 30.2$–32.9 min. This eluate was purified on the same column with 35% methanol in 1% acetic acid (aq.) at a flow rate of 2.0 ml/min, and compound **2** was isolated at $R_t = 11.2$ min.

*Isolation of Compound **3**.* Compound **3** [quercetin 3-*O*-(2^G-β-D-xylopyranosylrutinoside)] was isolated as described by Nishida et al. (1990).

*Acid Hydrolysis of Compounds **1** and **2**.* Compounds **1** and **2** (each 500 μg) were dissolved in 1 N HCl (500 μl) and held at 90°C for 12 hr, respectively. Each reaction mixture was extracted with ethyl acetate and washed with saturated NaCl. The organic layer was dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to give the aglycone of each compound.

*Preparation of the Peracetyl Derivative of Compound **1**.* A solution of compound **1** (10 mg) in pyridine (1 ml), was treated with acetic anhydride (1 ml), and the mixture was stirred at 70°C for 12 hr. The reaction mixture was dissolved in ethyl acetate and washed successively with 0.1 N HCl and saturated NaCl. The organic layer was dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to give the peracetyl derivative (12 mg).

Instruments for Chemical Analysis. Preparative HPLC was carried out with an Altex 110 pump, monitoring eluates with a differential refractometer, model R401 (Waters Associates). High-resolution fast atom bombardment mass spectrometry (HR-FAB-MS) was carried out with a JEOL HX211A mass spectrometer. ^{1}H and ^{13}C NMR spectra were measured with Bruker AC300 FT-NMR and ARX500 FT-NMR spectrometers using TMS as a standard. Optical rotation was measured with a JASCO DIP-370 spectropolarimeter. UV spectra were measured with a Beckman DU-64 Spectrophotometer.

RESULTS

Fractionation and Deterrent Activity. A methanolic extract of fresh leaves of *O. japonica* was partitioned between water and ethyl acetate. The ethyl acetate layer showed moderate oviposition deterrent activity (56.3% at a dose of 0.1 g.l.e./f.p., $N = 32$) but was inactive in the feeding deterrent bioassay (0% at a dose of 0.1 g.l.e./t.l., $N = 40$). The water layer showed distinct deterrent activities

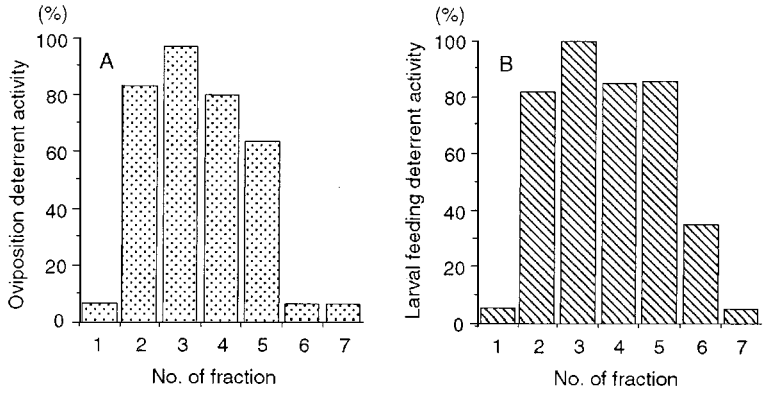


FIG. 2. Deterrent activities of ODS-column chromatographic fractions of *Orixa japonica* extract to *Papilio xuthus*. (A) Oviposition deterrent activities at 0.1-g leaf equivalent/filter paper ($N = 30$); (B) larval feeding deterrent activities at 0.3-g leaf equivalent/test leaflet ($N = 20$–22).

in both oviposition (85.7% at a dose of 0.1 g.l.e./f.p., $N = 35$) and larval feeding (97.5% at a dose of 0.3 g.l.e./t.l., $N = 40$) bioassay. The water layer was separated on a reverse phase column into seven fractions, of which Fr. 2–5 showed potent deterrent activities both for oviposition and larval feeding (Figure 2). Fr. 2 and Fr. 3 were further fractionated by HPLC, and active compounds **1** and **2** were isolated (**1**: a white powder, 25 μg from 450-g fresh leaves; **2**: a colorless syrup, 11 μg from 1500-g fresh leaves). Compound **3**, reported as one of the oviposition deterrents for *P. xuthus* (Nishida et al., 1990), was isolated from Fr. 4 (30 mg/200 g fresh leaves). Thus, deterrent assays were conducted at the following doses based on the contents per gram leaf equivalent: compound **1**, 56 μg; **2**, 7.3 μg; **3**, 150 μg, respectively.

Compound **1** showed strong oviposition deterrent (71.4% at a dose of 0.1 g.l.e./f.p., $N = 35$) and feeding deterrent (68.0% at a dose of 0.3 g.l.e./t.l., $N = 31$) activities sufficient to account for the deterrence of Fr. 2: Oviposition deterrent activity was not significantly different at a dose of 0.1 g.l.e./f.p. ($P > 0.05$, χ^2 test, $N = 30$–35), while feeding deterrent activity did not differ at a dose of 0.3 g.l.e./t.l. ($P > 0.05$, χ^2 test, $N = 22$–31). Although compound **2** also showed strong oviposition deterrence (73.3% at a dose of 0.1 g.l.e./f.p., $N = 30$), the activity was significantly lower than that of Fr. 3 ($P < 0.05$, χ^2 test, $N = 30$). On the other hand, compound **2** showed strong feeding deterrent activity (84.0% at a dose of 0.3 g.l.e./t.l., $N = 25$), which appeared to account for that of Fr. 3 [NSD (no significant difference) at a dose of 0.3 g.l.e./t.l., $P > 0.05$, χ^2 test, $N = 25$]. Compound **3** showed only weak oviposition deterrent activity (24.0% at a dose of 0.1 g.l.e./f.p., $N = 25$) and little feeding deterrent activity (15.7% at a dose of 0.3 g.l.e./t.l., $N = 38$).

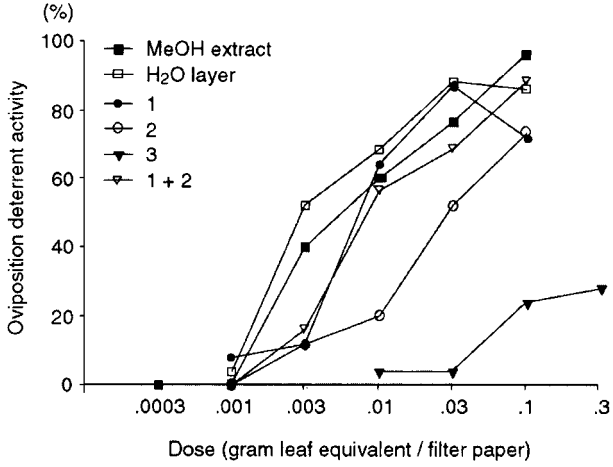


FIG. 3. Dose–response relationships of extracts of *Orixa japonica* and the isolated compounds in the oviposition bioassay for *Papilio xuthus* ($N = 25$–35).

Dose responses of test samples for female oviposition deterrent activity and larval feeding deterrent activity are shown in Figures 3 and 4, respectively. The water layer was as active as the methanolic extract in the oviposition bioassay (NSD at all doses, $P > 0.05$, χ^2 test, $N = 25$–35). The activity of compound **1**

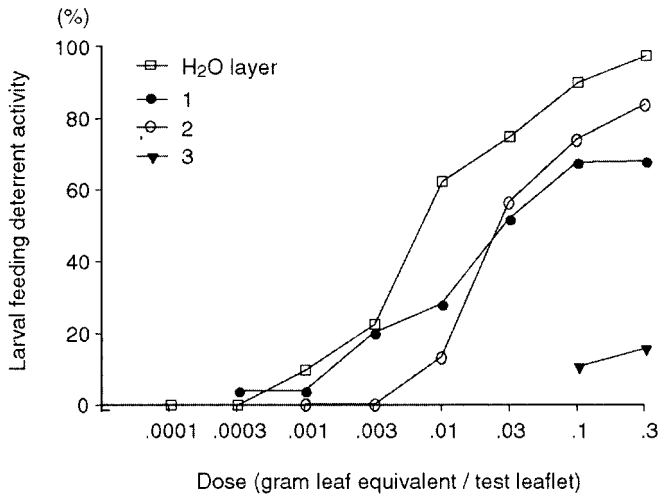
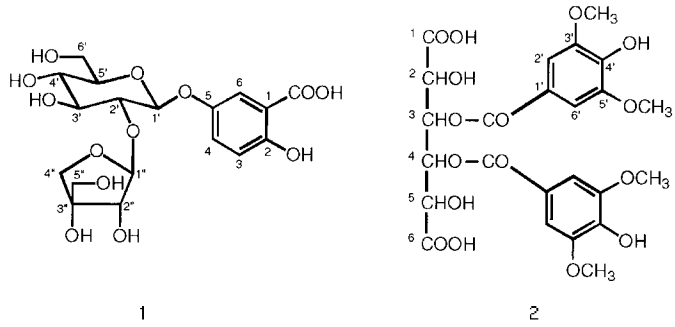


FIG. 4. Dose–response relationships of an aqueous extract of *Orixa japonica* and the isolated compounds in the feeding bioassay for fifth instar larvae of *Papilio xuthus* ($N = 23$–40).

was similar to that of the water layer (NSD at doses of 0.001, 0.01, 0.03, 0.1 g.l.e., $P > 0.05$, χ^2 test, $N = 25$–35). Compounds **1** and **2** did not appear to synergize each other (Compound **1** + **2**: not significantly different from compound **1** alone at all doses, $P > 0.05$, χ^2 test, $N = 25$–35; not significantly different from compound **2** alone at doses of 0.001, 0.003, 0.03, 0.1 g.l.e., $P > 0.05$, χ^2 test, $N = 25$–35). For larval feeding deterrent activity, both compounds **1** and **2** showed strong activity, but did not account for that of the original water layer.

*Structure of Compound **1***. The molecular formula of **1** was determined to be $C_{18}H_{24}O_{13}$ from the HR-FAB-MS. Acid hydrolysis of **1** afforded gentisic acid. The ^{13}C NMR spectrum of **1** exhibited 11 carbon signals ascribable to a diglycosidic moiety including two anomeric carbons (δ 111.0 and 102.3) in addition to those of the gentisic acid moiety. The ^{1}H–^{1}H COSY spectrum of **1** revealed a sequential *trans*-1,2-diaxial relationship of H-1′/H-2′/H-3′/H-4′/H-5′ ($J = 6.9$–7.6 Hz) and two correlated methylene signals (H-6′*a*, *b*) indicated the presence of a glucopyranosyl moiety in **1**. The remaining pentose moiety was established to be apiose by ^{1}H and ^{13}C NMR analysis, where (1) the anomeric proton (H-1″) was coupling vicinally with H-2″ ($J = 1.6$ Hz); (2) two pairs of isolated methylenes were observed as geminal AB-type doublets (H-4″*a*, *b*, $J = 9.6$ Hz) and a singlet (H-5″); (3) HMBC correlations were observed between H-1″ and C-3″ and between H-1″ and C-4″. The HMBC spectrum also exhibited correlations between H-1″ of apiose and C-2′ of the glucopyranosyl moiety and between glucopyranosyl H-1′ and C-5 of the gentisic acid. The ^{1}H NMR spectrum of a peracetyl derivative of **1** exhibited the H-2′ signal at δ 3.92 that indicated the apiosyl moiety was connected to the glucosyl C-2′ position. Since apiose is known to be biosynthesized from D-glucose via decarboxylation of UDP-D-glucuronic acid (Watson and Orenstein, 1975), both sugar components in **1** were deduced to be a D-form. Further, by applying Klyne's rule to the glycoside (Klyne, 1950), the apiofuranose was suggested to possess a β-D-furanosyl form, because **1** gave a negative molecular rotation ($[\phi]_D$ −385°) [cf. gentisic acid 5-*O*-β-D-glucopyranoside: $[\phi]_D$ −168° (Yahara et al., 1985); methyl β-D-apiofuranoside: $[\phi]_D$ −156° (Kitagawa et al., 1993)]. Therefore, compound **1** was identified as 5-{[2-*O*-(β-D-apiofuranosyl)-β-D-glucopyranosyl]oxy}-2-hydroxybenzoic acid (Figure 5), which was previously reported as a constituent of *Mimosa pudica* (Fabaceae) (Hettinger and Schildknecht, 1984).

*Compound **1***: $[\alpha]_D^{22}$ − 86.0°($c = 0.5$, MeOH); UV (MeOH) λ_{max} nm (log ε): 233 (3.78), 322 (3.52); ^{1}H NMR (CD_3OD) aglycon δ 7.57 (1H, d, $J = 3.0$ Hz, H-6), 7.26 (1H, dd, $J = 9.0$, 3.0 Hz, H-4), 6.85 (1H, d, $J = 9.0$ Hz, H-3); sugars, Glc δ 4.84 (1H, d, $J = 6.9$ Hz, H-1′), 3.88 (1H, dd, $J = 12.1$, 1.7 Hz, H-6′*a*), 3.70 (1H, dd, $J = 12.1$, 4.8 Hz, H-6′*b*), 3.60 (1H, dd, $J = 7.3$, 6.9 Hz, H-2′), 3.59 (1H, dd, $J = 7.6$, 7.3 Hz, H-3′), 3.40 (1H, ddd, $J = 7.6$, 4.8, 1.7, Hz, H-5′), 3.39 (1H, dd, $J = 7.6$, 7.6 Hz, H-4′); Api δ 5.45 (1H, d, $J = 1.6$ Hz, H-1″), 4.06 (1H, d, $J = 9.6$ Hz, H-4″*a*), 3.98 (1H, d, $J = 1.6$ Hz, H-2″), 3.79 (1H, d, $J = 9.6$ Hz, H-4″*b*), 3.57 (2H, s, H-5″); ^{13}C NMR (CD_3OD) aglycon δ 173.4 (COOH), 158.7



1 2

FIG. 5. Oviposition and feeding deterrents for *Papilio xuthus* identified from *Orixa japonica*. **1**: 5-{[2-*O*-(β-D-apiofuranosyl)-β-D-glucopyranosyl]oxy}-2-hydroxybenzoic acid; **2**: 3,4-*O*-disyringoyl-aldaric acid.

(C-2), 151.3 (C-5), 126.6 (C-4), 118.9 (C-6), 118.8 (C-3), 114.3 (C-1); sugars, Glc δ 102.3 (C-1′), 79.1 (C-3′), 78.6 (C-2′), 78.0 (C-5′), 71.4 (C-4′), 62.5 (C-6′); Api δ 111.0 (C-1″), 80.7 (C-3″), 78.2 (C-2″), 75.4 (C-4″), 66.0 (C-5″); HR-FAB-MS (negative mode, triethanolamine): m/z 447.1154$[M-H]^-$, calcd for $C_{18}H_{23}O_{13}$, 447.1138).

*Aglycone of **1** (gentisic acid)*: ^{1}H NMR (CD_3OD) δ 7.24 (1H, d, $J = 3.1$ Hz, H-6), 6.95 (1H, dd, $J = 8.9$, 3.1 Hz, H-4), 6.76 (1H, d, $J = 8.9$ Hz, H-3).

*Compound **1** Hexa-acetate*: ^{1}H NMR ($CDCl_3$) aglycone δ 7.58 (1H, d, $J =$ 3.1 Hz, H-6), 7.27 (1H, dd, $J = 9.1$, 3.1 Hz, H-4), 6.94 (1H, d, $J = 9.1$ Hz, H-3); sugars Glc δ 5.27 (1H, dd, $J = 9.4$, 9.4 Hz, H-3′), 5.04 (1H, dd, $J = 9.8$, 9.4 Hz, H-4′), 4.89 (1H, d, $J = 7.7$ Hz, H-1′), 4.26 (1H, dd, $J = 12.3$, 5.5 Hz, H-6′), 4.15 (1H, dd, $J = 12.3$, 2.4 Hz, H-6'), 3.92 (1H, dd, $J = 9.4$, 7.7 Hz, H-2′), 3.81 (1H, ddd, J = 9.8, 5.5, 2.4 Hz, H-5′); Api δ 5.20 (2H, H-1″ and H-2″), 4.60 (2H, s, H-5″), 4.36 (1H, d, $J = 10.3$ Hz, H-4″), 4.14 (1H, d, $J = 10.3$ Hz, H-4″); AcO δ 2.12 (s), 2.10 (s), 2.08 (s), 2.05 (s), 2.05 (s), 2.04 (s).

*Structure of Compound **2***. The molecular formula of compound **2** was determined to be $C_{24}H_{26}O_{16}$ from the HR-FAB-MS analysis. Acid hydrolysis of **2** afforded syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid). The ^{1}H NMR spectrum revealed two pairs of isolated aromatic proton signals (δ 7.15 and 7.14) and two pairs of methoxy signals (δ 3.812 and 3.807) arising from two syringoyl moieties. The ^{13}C NMR signals for the two syringoyl moieties ($2 \times C_9H_9O_4$) were assigned by the aid of ^{1}H–^{1}H COSY, HMQC, and HMBC correlations and comparison with these of authentic syringic acid as shown in Table 1. The remaining moiety ($C_6H_8O_8$) was assigned as an aldaric acid from the 2D NMR analysis as follows: The ^{1}H–^{1}H COSY spectrum showed four sequentially arrayed *O*-methine protons at δ 4.73 (H-2), 6.15 (H-3), 5.93 (H-4), and 4.64 (H-5) ($J_{2-3} = 2.2$ Hz, $J_{3-4} = 9.0$ Hz, and $J_{4-5} = 2.7$ Hz). The HMQC and HMBC spectra revealed the four corresponding methine carbon signals at δ 71.1 (C-2), 74.7 (C-3), 75.9

TABLE 1. ^{13}C NMR ASSIGNMENTS OF COMPOUND **2** AND SYRINGIC ACID IN CD_3OD

Moiety	Compound **2**	Syringic acid
Aldaric acid		
C-1	174.6	
C-2	71.1	
C-3	74.7	
C-4	75.9	
C-5	71.7	
C-6	174.6	
Syringoyl		
COO	167.3, 167.1	170.0
C-1′	121.1 (2C)	121.9
C-2′, C-6′	108.7 (2C), 108.6 (2C)	108.4 (2C)
C-3′, C-5′	148.7 (4C)	148.9 (2C)
C-4′	141.9 (2C)	141.8
OCH_3	56.9 (2C), 56.8 (2C)	56.8 (2C)

(C-4), and 71.7 (C-5), and a signal arising from two carboxylic carbons at δ 174.6 (C-1 and C-6). Since HMBC correlations were observed between the two syringoyl carbonyl carbons (δ 167.3 and 167.1) and methine protons of H-3 and H-4, respectively, attachment of the syringoyloxy moieties to the C-3 and C-4 positions of the aldaric acid was verified. Therefore, the structure of compound **2** can be given tentatively by the formula as shown in Figure 5 as a 3,4-*O*-disyringoyl aldaric acid with unknown stereochemistry. Among the 10 possible stereoisomers of aldaric acids, as to four chiral centers (C-2, 3, 4, and 5), only (+) or (−)-glucaric or (+) or (−)-altraric acids suit the structure of compound **2** because of the chiral nature. Further experiments are needed to clarify the stereochemistry.

*Compound **2***: $[\alpha]_D^{22} - 65.8^\circ$ ($c = 1.0$, MeOH); UV (MeOH) λ_{max} nm (log ε): 278 (3.69); ^{1}H NMR (CD_3OD) syringoyl moiety δ 7.15 (2H, s), 7.14 (2H, s), 3.812 (6H, s, 2 × CH_3O), 3.807 (6H, s, 2 × CH_3O); aldaric acid moiety δ 6.15 (1H, dd, $J = 9.0$, 2.2 Hz, H-3), 5.93 (1H, dd, $J = 9.0$, 2.7 Hz, H-4), 4.73 (1H, d, $J = 2.2$ Hz, H-2), 4.64 (1H, d, $J = 2.7$ Hz, H-5); ^{13}C NMR (see Table 1); HR-FAB-MS (positive mode, glycerol): m/z 571.1284 $[M + H]^+$, calcd for $C_{24}H_{27}O_{16}$, 571.1299).

*Hydrolysis product of **2**(syringic acid)*: ^{1}H NMR (CD_3OD): δ 7.33 (2H, s, H-2, 6), 3.88 (6H, s, 2 × CH_3O).

DISCUSSION

We characterized compounds **1** and **2** from a nonhost rutaceous plant, *O. japonica*, as the major oviposition and larval feeding deterrents against *P. xuthus*.

While compound **1** showed strong oviposition deterrent activity, which accounted for that of Fr. 2 and the parent aqueous fraction, compound **2** showed relatively weak activity that accounted for only a part of the activity of Fr. 3. This suggested the presence of one or more additional oviposition deterrents in this fraction. On the other hand, both compounds **1** and **2** accounted fully for the feeding deterrent activities of Fr. 2 and Fr. 3, respectively. In *Manduca sexta* larvae, both gustation and olfaction are important for host-plant discrimination (Hanson and Dethier, 1973), while rejection of non-host-plant extract is mediated by taste receptor cells in the maxillary palps (Glendinning et al., 1998). Likewise, *P. xuthus* larvae exhibited an immediate rejection response after few trial bites on the test leaflet treated with *Orixa* extract. The highly polar and nonvolatile nature of compounds **1**–**3** suggests that these compounds function as "deterrents" through contact chemoreceptors on both oviposition and larval feeding (Dethier et al., 1960). The flavonoid triglycoside quercetin 3-*O*-(2^{G}-β-D-xylopyranosylrutinoside) (**3**), previously identified as an oviposition deterrent for *P. xuthus* (Nishida et al., 1990), showed only weak activity compared to those of compounds **1** and **2** (Figure 3). Compound **3** may "disrupt" the oviposition stimulant activity of the *Citrus* extract due to its relatively high concentrations in the test samples or perhaps to its structural similarity to rutin, one of the oviposition stimulants for *P. xuthus* (Nishida et al., 1987). Although rutin was found as a constituent of *O. japonica* leaves, the presence or lack of other oviposition or feeding stimulants remains to be clarified.

Compounds **1** and **2** were identified as the gentisic acid ester of a diglycoside and the disyringoyl ester of an aldaric acid, respectively. Although these compounds are not closely related to each other, both are classified as hydroxybenzoic esters of sugar derivatives. Compound **1** has been reported only from *M. pudica* (Fabaceae) (Hettinger and Schildknecht, 1984). Gentisic acid 5-*O*-β-D-glucopyranoside, a compound related to **1** and isolated from Fr. 2, showed little deterrent activity for female oviposition. However, apiin (7-{[2-*O*-(β-D-apiofuranosyl)-β-D-glucopyranosyl]oxy}-apigenin), a major constituent of plants in the family Apiaceae, has the same glycosidic (apiosyl–glucosyl) linkage as **1** and showed moderate deterrent activity (data not shown). The sugar moiety may, therefore, contribute to the oviposition deterrent properties of such compounds for *P. xuthus*. Likewise, the oviposition deterrent activity of cardenolides has been shown to be strongly dependent on the sugar moiety in *Pieris rapae* (Sachdev-Gupta et al., 1990). It remains unclear, though, whether compound **1** blocks the intrinsic activity of oviposition stimulative flavonoids or exerts its effect by other mechanisms. Compound **2** is a unique disyringoyl ester of an aldaric acid that has not been reported previously from plants.

Although deterrents play an important role in ovipositional and feeding behaviors in the family Papilionidae, only a few deterrent chemicals have so far been identified. Flavonoid glycosides reported as oviposition deterrents include compound **3**

from *O. japonica* against *P. xuthus* (Nishida et al., 1990) and phellamurin against *P. protenor* (Honda and Hayashi, 1995b). For larval feeding, a neolignoid asatone was identified as a feeding deterrent against larvae of an Aristolochiaceae-feeding specialist *Luehdorfia puziloi* from a non-host aristolochiaceous plant, *Heterotropa aspera* (Honda et al., 1995). It has been shown that a specific flavonoid glycoside can act as either a stimulant or a deterrent for different papilionid species: Phellamurin deters oviposition of *P. protenor*, while the same compound stimulates oviposition of *P. maakii* (Honda et al., 1997). Hydroxybenzoic acid derivatives **1** and **2** are structurally different from a series of known oviposition stimulants (or deterrents) for papilionid butterflies.

There are two *Papilio* species that utilize *O. japonica* as one of their major hosts. Oviposition stimulants of these *Orixa*-feeders, *P. macilentus* and *P. bianor*, have been partly elucidated (Nishida, 1995; Ono et al., 2000a,b). In these cases, oviposition behavior was triggered by multiple factors, each of which elicited specific responses either singly or synergistically. Both oviposition and feeding deterrents **1** and **2** in *O. japonica* against *P. xuthus* showed distinct activity independently without apparent synergism between the compounds. It is not known whether **1** and **2** have any behavioral effects on the above *Orixa*-feeders.

Leaves of *O. japonica* contain a series of noxious compounds such as furanocoumarins (isopimpinellin, bergapten, and xanthotoxin) and quinoline alkaloids (kokusagine, evoxine, and japonin) that inhibit feeding of an armyworm, *Spodoptera liture* (Yajima et al., 1977). This generalist larva may have attained a sensory ability to avoid these chemicals before ingestion. It is yet uncertain whether or not these allomonal or toxic chemicals were produced in response to herbivorous attack and/or the insects gained the gustatory sensitivity to avoid potential toxins before ingestion through their coevolutionary interactions. *P. macilentus* and *P. bianor*, which are highly specialized to *O. japonica*, may provide clues to mechanisms of adaptation against these allelochemicals.

Physiological studies have characterized chemoreceptors related to host selection. For female tarsal chemoreceptors, several stimulants and deterrents stimulate specific receptor cells in the medial sensilla of several oligophagous butterflies such as *Pieris* butterflies (Ma and Schoonhoven, 1973; Du et al., 1995; Städler et al., 1995), *Papilio polyxenes* (Roessingh et al., 1991), and *Danaus plexippus* (Bauer et al., 1998). As for larval chemoreception, the presence of two types of deterrent chemoreceptors has been indicated: generalist deterrent receptors responsive to a broad spectrum of plant allelochemicals and more specialized receptors sensitive to specific deterrents such as cardenolides (van Loon and Schoonhoven, 1999). Coincidence in utilization of specific classes of deterrents chemicals on both oviposition and larval feeding has been demonstrated in *P. rapae*, where cardiac glycosides in non-host crucifers are responsible for the rejection of the plant even though the compounds sensed by the adults at oviposition and larvae at feeding did not show an exact match (Sachdev-Gupta et al., 1990, 1993). In contrast,

our results suggest the coincidence in oviposition deterrence and larval feeding deterrence might be controlled by a common sensory mechanism in the tarsal chemoreceptors of adults and the gustatory chemoreceptors of larvae. Therefore, it is intriguing to investigate the correlation between adult and larval chemoreception at a receptor level, which may show us the molecular evidence of sensory adaptation at the insect–plant interface.

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RAPID HERBIVORE-INDUCED CHANGES IN MOUNTAIN BIRCH PHENOLICS AND NUTRITIVE COMPOUNDS AND THEIR EFFECTS ON PERFORMANCE OF THE MAJOR DEFOLIATOR, *Epirrita autumnata*

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Abstract—Insect damage changes plant physiology and chemistry, and such changes may influence the performance of herbivores. We introduced larvae of the autumnal moth (*Epirrita autumnata* Borkh.) on individual branches of its main host plant, mountain birch (*Betula pubescens* ssp. *czerepanovii* (Orlova) Hämet-Ahti) to examine rapid-induced plant responses, which may affect subsequent larval development. We measured systemic responses to herbivory by analyzing chemistry, photosynthesis, and leaf growth, as well as effects on larval growth and feeding, in undamaged branches of damaged and control trees. Larvae reared on leaves from intact branches of the herbivore-damaged trees grew faster than those reared on leaves of control trees, indicating systemic-induced susceptibility. Herbivore damage did not lead to systemic changes in levels of primary nutrients or phenolic compounds. The analyses of photosynthetic activity and individual hydrolyzable tannins revealed a reversal of leaf physiology-herbivore defense patterns. On control trees, consumption by *E. autumnata* larvae was positively correlated with photosynthetic activity; on damaged trees, this correlation was reversed, with consumption being negatively correlated with photosynthetic activity. A similar pattern was found in

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the relationship between monogalloylglucose, the most abundant hydrolyzable tannin of mountain birch, and leaf consumption. Among the control trees, consumption was positively correlated with concentrations of monogalloylglucose, whereas among herbivore-damaged trees, this correlation was reversed and became negative. Our results suggest that herbivore performance is related to both concentrations of phenolic compounds and photosynthetic activity in leaves. This linkage between herbivore performance, leaf chemistry, and physiology was sensitive to induced plant responses caused by slight herbivore damage.

Key Words—*Epirrita autumnata*, *Betula pubescens* ssp. *czerepanovii*, galloylglucoses, larval growth, leaf consumption, Lepidoptera, photosynthesis, rapid-induced responses.

INTRODUCTION

Herbivory modifies plant growth and physiology, including chemistry, which may change plant quality for subsequently attacking herbivores (induced resistance and susceptibility) (Karban and Baldwin, 1997). The bulk of current research in this area concentrates on induced resistance, in part because delayed forms of induced resistance (DIR), affecting the following generation of herbivores, offer a mechanism that may contribute to cycles in herbivore populations.

In the mountain birch (*Betula pubescens* ssp. *czerepanovii*) – autumnal moth system, herbivore-induced responses in host leaf quality are not restricted to DIR; intact birch leaves tend to be of lower quality to *E. autumnata* within days after manual damage to nearby leaves (i.e., rapid-induced resistance, RIR) (Haukioja and Hanhimäki, 1985; Hanhimäki and Senn, 1992). These rapid-induced changes also have the potential to influence population dynamics. For example, there are indications that damage to birch buds and shoots makes foliage more palatable for later herbivory (Haukioja et al., 1990; Danell et al., 1997). Such induced susceptibility is a potentially important phenomenon for population cycles because it might introduce positive feedback into the population dynamics of the herbivore, which could explain the elusive increase phase of outbreaking herbivores (Ruohomäki et al., 2000).

The mechanisms by which birch trees show induced resistance and susceptibility are extensively studied. Birch leaves have both physical (leaf toughness) and chemical traits that may serve defensively against insect herbivores (Ruohomäki et al., 1996; Kause et al., 1999; Ossipov et al., 2001). Levels of total nitrogen and total phenolics are known to change after defoliation (Neuvonen and Haukioja, 1984; Haukioja et al., 1985; Ruohomäki et al., 1996; Kaitaniemi et al., 1998), making a potential link between plant responses and resistance to herbivores. There is some data demonstrating local RIR on *E. autumnata* after manual damage to leaves, and also of systemic RIR after larval damage (Kaitaniemi and Ruohomäki, 2001). To further test for the existence and chemical correlates of possible systemically

induced effects after real herbivore damage on the performance of *E. autumnata* larvae, we conducted a field experiment by introducing larvae to trees in a natural population.

Recent investigations have revealed large differences in seasonal patterns among different groups of phenolic compounds and carbohydrates and amino acids, the main pool of nitrogen-rich compounds in birch (Kause et al., 1999; Riipi et al., 2002). Among phenolic compounds, proanthocyanidins increase during leaf expansion and maturation, whereas flavonoids and galloylglucoses, a group of hydrolyzable tannins, decrease with phenology (Salminen et al., 1999, 2001; Riipi et al., 2002). Thus, because our experiment followed the seasonal development of *E. autumnata* larvae on damaged and undamaged trees, we were able to examine seasonal patterns in the expression of plant resistance traits and how they were modified by herbivory.

Specifically, we addressed three main questions: (1) Do chemical, physical, and physiological traits of birch rapidly and systemically respond to herbivory? (2) Do responses in such traits affect consumption by or growth of *E. autumnata*? (3) Does herbivore damage to trees modify correlations among putatively defensive leaf traits, or between leaf traits and insect performance? To answer these questions we measured responses over the entire larval period of *E. autumnata* and measured larval performance during each instar.

METHODS AND MATERIAL

Study Organisms. Mountain birch (*Betula pubescens* spp. *czerepanovii*) is a northern European hardwood species forming uniform stands at the arctic tree line in northern Fennoscandia. There are large chemical changes in foliage during leaf unfolding and maturation (Ossipov et al., 1997, 2001; Riipi et al., 2002).

The autumnal moth (*Epirrita autumnata*) is a univoltine lepidopteran species and the main defoliator of mountain birch in NW Europe, with significantly cyclic regional peaks at 10-year intervals (Ruohomäki et al., 2000).

Experimental Design. We haphazardly selected 24 trees at a site situated in a river valley close to the Kevo Subarctic Research Station (69°45′ N, 27°01′ E). Twelve trees were randomly assigned to the larval introduction (herbivory) treatment, while 12 were left as unmanipulated controls. We used large (height 3–5 m) and mature mountain birch containing at least five large ramets with well-developed canopy. In each experimental tree, we selected five large ramets and two branches per ramet. The branches in the treatment trees were bagged with large (30 × 80 cm) nylon mesh bags (10 per tree) before bud burst in late May 2000, and we introduced approximately 20 larvae per bag during the natural egg hatch at bud burst. The mesh bags prevented larval dispersal and excluded parasitoids. We did not find wild *Epirrita* in the study site, and in general, there was negligible amount

of background herbivory. Although control trees were not bagged, in a separate experiment, we demonstrated that bags did not change suitability of the control trees to *E. autumnata*. In a laboratory bioassay, the means for the fifth instar larvae RGR reared with leaves of unbagged and bagged (empty) trees were 0.294 and 0.272 mg/d, respectively; $F_{1,18} = 1.19$, $P = 0.29$).

Leaf phenology is an important covariate of leaf resistance and herbivore performance, since delayed budbreak has deleterious effects on herbivore performance (Kaitaniemi et al., 1997). Therefore, at an early phase during the bud burst period, we measured leaf phenology as the ratio between the visible part of leaf blade and bud scale for each tree (Sulkinoja and Valanne, 1987).

Bioassays. We conducted laboratory bioassays by employing 20 larvae for each of the 24 trees, (480 larvae in total). The larvae were reared under natural light and temperature conditions out of doors, individually in 48-ml plastic vials. Larvae were fed from hatch to pupation with short shoot leaves detached from the experimental trees. Leaves were harvested from the short shoots in the branches without mesh bags. The order of vials was randomized in trays. We conducted bioassays with the experimental larvae in the second through fifth instar; the first instar larvae is too small and delicate to be successfully used in a bioassay. To allow natural variation in development, we did not synchronize development of experimental larvae before the bioassays. Therefore, all larvae could not be used in a given bioassay because some were molting. The numbers of larvae in the second, third, fourth, and fifth instar bioassay trials were 383, 369, 355, and 350, respectively. Although there was some variation, most larvae were individually followed throughout their development and employed in four bioassays.

The timing of the bioassays was decided by the developmental stage of the defoliating larvae reared in the bags of the experimental trees in the field. As soon as the mode number of experimental larvae molted into the particular instar, we conducted the bioassay. A 48-hr bioassay was used for the second and the third instar larvae, while a 24-hr one was used for the more voracious fourth and fifth instar larvae. Leaves for the bioassays were picked from experimental trees and stored in vials in a cooler until used. Each larva and leaf was weighed before the experiment. During the bioassays, larvae were kept in a temperature-controlled room at 12°C and in continuous light (average conditions at our high latitude study site). At the end of the bioassay, larvae were reweighed and leaf remnants and frass were collected. We freeze-dried leaf remnants for 48 hr before weighing. Larval fresh weight was converted into dry weight with the equation dry weight = $0.125 \times (\text{fresh weight})^{1.113}$ (Neuvonen and Haukioja, 1984). For each larva, we computed dry weight in the beginning of the bioassay and the amount of leaf consumed on a dry weight basis. During the bioassay, we collected 10 leaves per tree, weighed them fresh, dried them in an oven at 60°C for 24 hr, and reweighed them dry. The tree-specific regression models between fresh and dry weight of individual leaves explained more than 99% of total variation. For each tree, the

regression equation derived from the tree-specific model was used to convert leaf fresh weight into dry weight.

Chemical Analyses. We sampled short shoot leaves from untreated branches of the ramets of each experimental tree for chemical analysis when we collected leaves for each of the bioassays. Leaves were placed in a cooler, transferred to the laboratory, and freeze-dried. Freeze-dried leaves were homogenized, and about 300 mg of the powder were suspended in 10 ml of 70% aqueous acetone, allowed to stand for 1 hr at room temperature with continuous stirring, and centrifuged for 10 min at 2500 *g*. The pellet was reextracted twice. The acetone extract was reduced to the aqueous phase by evaporation at room temperature, and the resulting aqueous phase was frozen and lyophilized. The lyophilized residue was redissolved in 6 ml of water and centrifuged for 20 min at 3000 *g*. This purified extract was used for the determination of soluble phenolics and carbohydrates. The acetone-insoluble residue was collected, lyophilized, and weighed, and was used for the determination of cell-wall-bound proanthocyanidins and protein amino acids.

Soluble and cell-wall-bound proanthocyanidins were analyzed by the method of Terrill et al. (1992), which was modified and optimized for proanthocyanidins from birch leaves (Ossipova et al., 2001). Low molecular mass phenolics (chlorogenic acid and flavonoid-glycosides) were analyzed with HPLC (Salminen et al., 1999).

Soluble carbohydrates (glucose, fructose, sucrose, and galactose) and inositol were quantified using a gas chromatographic method (Kallio et al., 1985). Proteins were hydrolyzed with 6 N HCl for 24 hr at 105°C, and protein amino acids were derivatized with 9-fluorenylmethyl chloroformate and analyzed by HPLC with a fluorescent detector (Bank et al., 1996). Detailed description of all methods used can be found in Salminen et al. (1999), Ossipov et al. (2001), and Ossipova et al. (2001).

We paid special attention to analysis of hydrolyzable tannins because they undergo rapid biosynthesis during *E. autumnata's* larval period. HPLC-ESI-MS analysis of individual galloylglucoses and ellagitannins was performed as in Salminen et al. (1999, 2001), except that differences in the ESI-MS performance between individual runs were standardized by using 6-bromo-2-naphthyl-β-D-glucopyranoside as an internal standard.

Leaf Biomass, Toughness, and Photosynthesis Measurements. We measured tree-specific fresh weights of separately collected short shoot leaves at the time of each of the bioassays (five leaves per tree). We also measured leaf toughness by using a force gauge penetrometer (Chatillon DFIS, Amtek Inc. Largo, FL). We took, two measurements per leaf, piercing only intervenal parts of the leaf blade. After the toughness measurements, leaves were dried in the oven at 60°C for 48 hr and reweighed to get leaf dry weight.

Photosynthetic activity changes rapidly during bud burst and subsequent leaf development (Valanne and Valanne, 1984; Larcher, 1995), and previous defoliation reduces photosynthetic activity in mountain birch (Hoogesteger and Karlsson, 1992). We measured photosynthesis (A_{net}, net assimilation rate) with a portable photosynthesis system (a closed model CIRAS-1, PPSystems, Hitchin, UK) for each tree twice, June 21 and 28, and once after the bioassays, on July 14. A tree-specific $A_{net}$is the mean of the three dates. The amount of photosynthetically active radiation (PAR) was controlled with an external light source (1000 mol/m^{-2}/s^{-2}), which is above the photon flux density of *Betula* (Ovaska et al., 1992; Oleksyn et al., 1998). To minimize environmental noise, we took five measurements per replicate tree. Moreover, we measured A_{net} in the morning hours when A_{net} is the most active (Larcher, 1995), and bioassay specific measurements were taken during two or more days.

Statistical Analysis. We calculated tree-specific means by date for leaf consumption, larval growth, leaf phenology, toughness, and photosynthesis. Moreover, we calculated the sum of the concentrations of the individual compounds for galloylglucoses, ellagitannins, flavonoids, proanthocyanidins, carbohydrates, and protein-bound amino acids. We used concentrations (mg/g) of compounds instead of total amounts, since concentration of a compound indicates the quality of a plant as a food for herbivores (Koricheva, 1999). We performed a set of repeated measures MANCOVAs (proc GLM, SAS, 1996) to study effects of larval feeding on birch foliage: chemistry, leaf growth, leaf toughness, *E. autumnata* growth, and consumption. The sampling date was regarded as a within-subject variable. The models tested statistical significance of larval introductions (herbivory, main effect), leaf and larval growth, as well as changes in biochemical and physical variables through the season (date, main effect) and effects of defoliation on seasonal change (interaction of herbivory and date). The leaf phenology index at bud burst was employed as a covariate. We used the Pearson product moment correlation coefficient (proc CORR, SAS, 1996) to analyze relationships between biochemical and physical variables with larval traits. To meet the assumptions of analysis of variance, the values for concentrations of ellagitannins and proanthocyanidins were square-root transformed.

RESULTS

Seasonal Changes and Effects of Leaf Chewing on Leaf Chemistry. Herbivory by *E. autumnata* larvae in the trees did not change concentrations of any of the measured phenolic traits: hydrolyzable tannins or their subgroups (galloylglucoses and ellagitannins), flavonoids, proanthocyanidins (condensed tannins), chlorogenic acid, soluble carbohydrates (sugars), or protein-bound amino acids (in MANCOVAs $P > 0.25$) . There were significant seasonal development changes

in the concentrations of all compounds except total ellagitannins (Figure 1). Total concentrations of galloylglucoses, as well as monogalloylglucose, the most abundant galloylglucose, increased between June 8 and 16 and declined between June 16 and 28. Among ellagitannins, a decline in concentrations of tellimagrandin I and II, casuarictin, and potentillin was detected between June 21 and 28. Among major groups of low-molecular weight phenolic compounds, concentrations of flavonoids declined during leaf development. Eight individual flavonoid compounds out of nine followed the same pattern (data not shown). Concentrations of proanthocyanidins and chlorogenic acid, the most abundant phenolic compound in mountain birch, displayed a steady increase during the experimental period. Among major primary metabolites, total concentration of carbohydrates increased during leaf development, but defoliation did not modify concentrations ($P = 0.39$; Figure 2). This pattern was consistently found among four individual carbohydrates, except sucrose, which peaked during the third instar of *E. autumnata* (data not shown). Total concentration of protein-bound amino acids decreased during leaf development, but defoliation did not change concentrations ($P = 0.78$; Figure 2).

Effects of Defoliation on Leaf Growth, Photosynthesis, and Toughness. Leaf biomass, photosynthetic activity, and leaf toughness all increased during the experimental period. Defoliation, however, did not affect these traits, suggesting that mountain birch did not respond systemically to the relatively small amount of damage imposed (Figure 3).

Effects of Rapid-Induced Responses on Larval Growth and Leaf Consumption. The presence of experimentally imposed herbivory by *E. autumnata* larvae systemically modified the growth of bioassay larvae during leaf development. The larvae reared on leaves from defoliated trees developed 16% faster than those on leaves from control trees. The growth curves set apart during the fourth instar, as indicated by a significant time $\times$ defoliation interaction (Figure 4, Table 1). Interestingly, defoliation did not affect pupal mass ($F_{1,\ 22} = 0.16$, $P = 0.69$), indicating that the slower growing larvae in control trees could feed longer, thus compensating for the difference in growth rates. Leaf consumption by *E. autumnata* naturally increased during larval development, but defoliation did not modify consumption patterns during leaf development (Table 1, Figure 4).

Chemical and Physiological Correlates of Larval Growth and Feeding. We conducted correlational analyses to reveal how the chemical and physiological quality of leaves was related to growth of, and consumption by *E. autumnata*. The main chemical compounds correlating with moth performance were concentrations of flavonoids, chlorogenic acid, and protein-bound amino acids. Flavonoids and chlorogenic acid were positively correlated with leaf consumption, whereas amount of protein-bound amino acids was positively related to pupal mass (Table 2).

Effects of Rapid-Induced Responses on Relationships Between Putative Defenses and Insect Performance. To understand how defoliation may affect

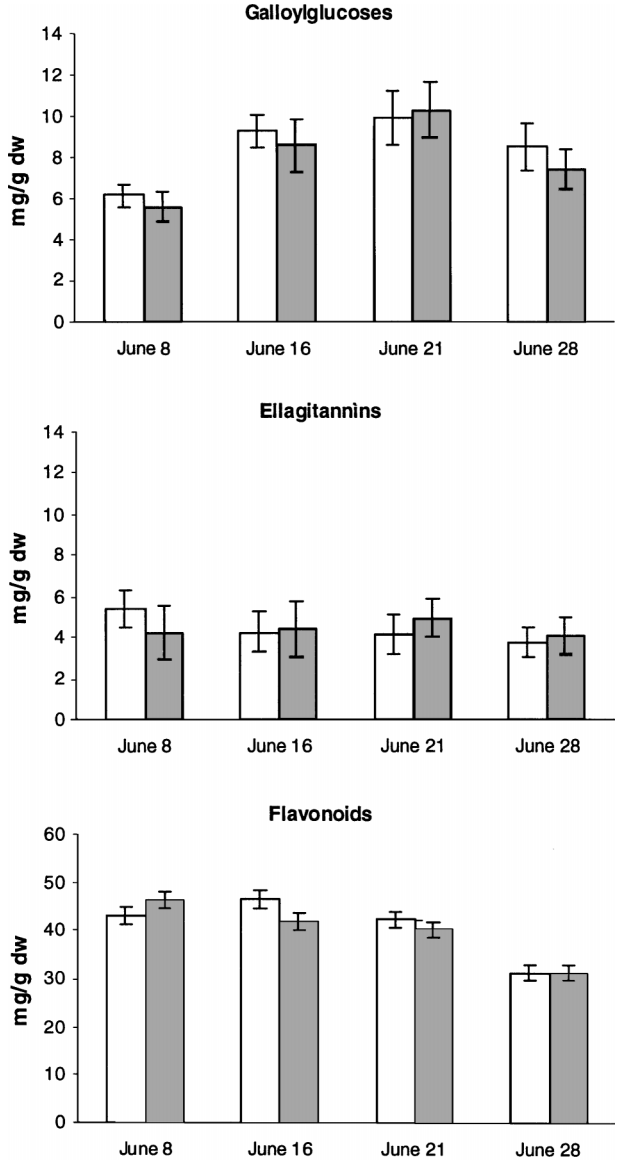

FIG. 1. Contents of ellagitannins, galloylglucoses, flavonoids, proanthocyanidins, and chlorogenic acid during the early phases of birch leaf development. The clear bars represent controls, and grey bars represent trees with experimentally imposed herbivory. Means and their standard errors are shown. Seasonal changes are statistically significant ($P < 0.01$) except for total ellagitannins ($P = 0.19$).

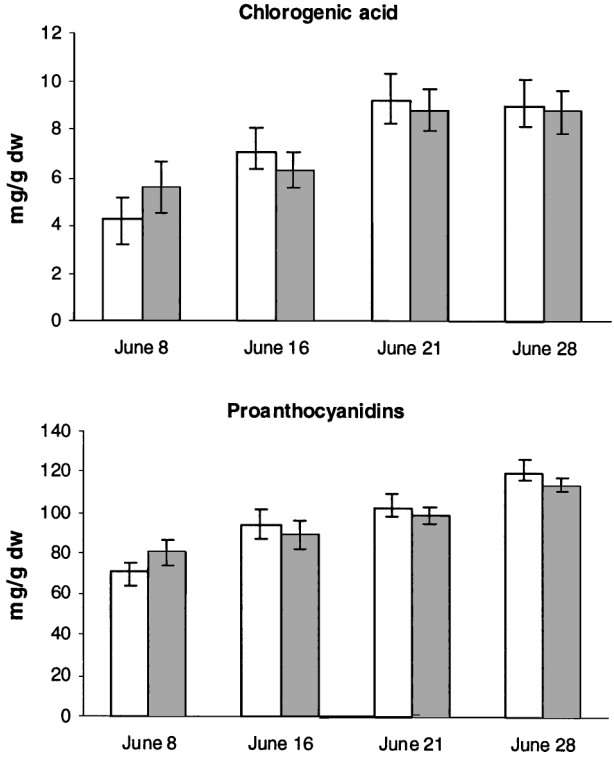

FIG. 1. CONTINUED.

relationships between foliar quality and herbivore performance, we conducted many ANCOVAs to explain herbivore growth and leaf consumption. As explanatory factors, we used herbivory treatment, foliar chemistry/physiology, and their interactions; leaf phenology was used as a covariate. A significant interaction term suggests that herbivory not only modified leaf suitability for larvae, but also the relationship between chemical and physiological quality and herbivore performance. After a sequential Bonferroni correction, we discovered two significant interactions: relationships of leaf consumption between photosynthesis and the simplest galloylglucose, monogalloylglucose, were different between control trees and herbivore-damaged trees. In the control trees, leaf consumption was positively correlated with photosynthetic activity, whereas in the defoliated trees, leaf consumption was negatively correlated (interaction term in ANCOVA: $F_{1,19} = 7.04$, $P = 0.015$, Figure 5). A similar pattern was found between monogalloylglucoses and leaf consumption (interaction term in ANCOVA: $F_{1,19} = 9.29$, $P = 0.006$, Figure 5). Such reversals of the correlations indicate a

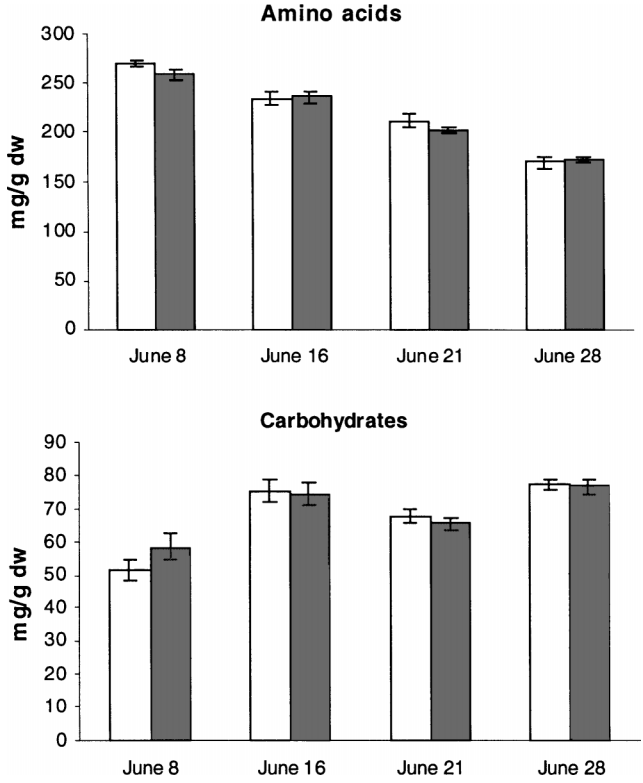

FIG. 2. Contents of amino acids and carbohydrates during the early phases of birch leaf development. The clear bars represent controls, and grey bars represent trees with experimentally imposed herbivory. Means and their standard errors are shown. Seasonal changes are statistically significant ($P < 0.01$).

fundamental change in photosynthetic activity, physiological basis of growth, and mode of action of monogalloylglucose, a putative defensive compound of mountain birch.

DISCUSSION

Our results show that *E. autumnata* grew better on leaves from trees damaged by *E. autumnata* larvae than on control trees, indicating rapid-induced susceptibility. Our goal was to create moderate levels of herbivory that would simulate the types of damage during the increase phase of an outbreak. The positive effect of the presence of damaging larvae (350 out of 480 larvae lived up to fifth instar)

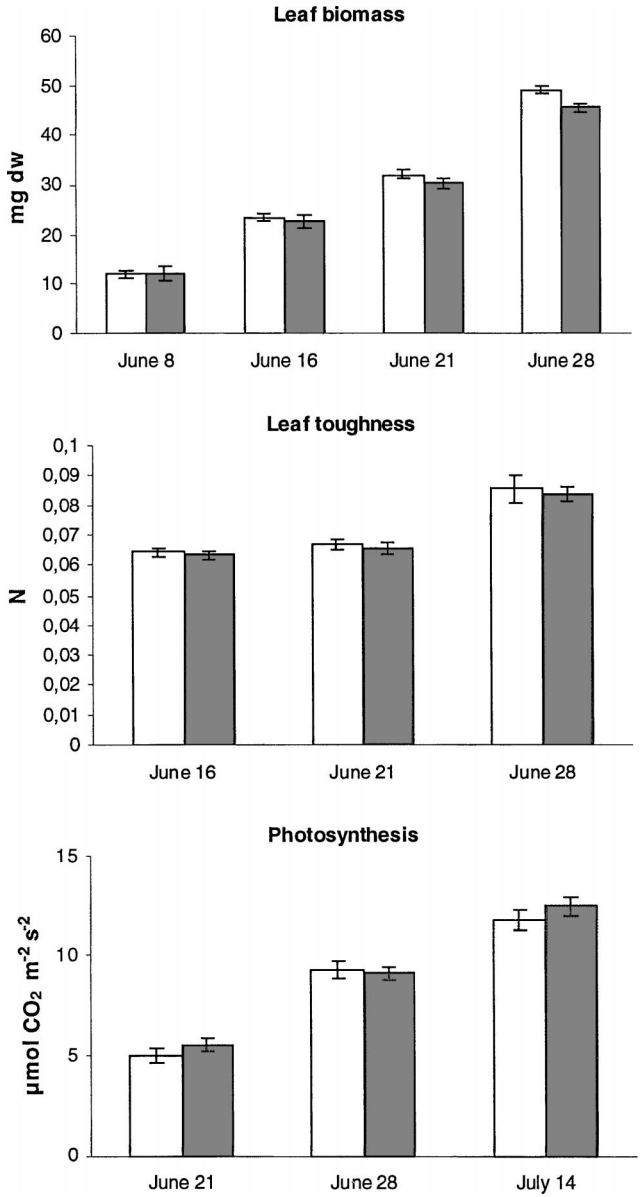

FIG. 3. Changes in leaf biomass, photosynthetic activity, and leaf toughness during birch leaf development. The clear bars represent controls, and grey bars represent trees with experimentally imposed herbivory. Means and their standard errors are shown. Seasonal changes are statistically significant ($P < 0.01$).

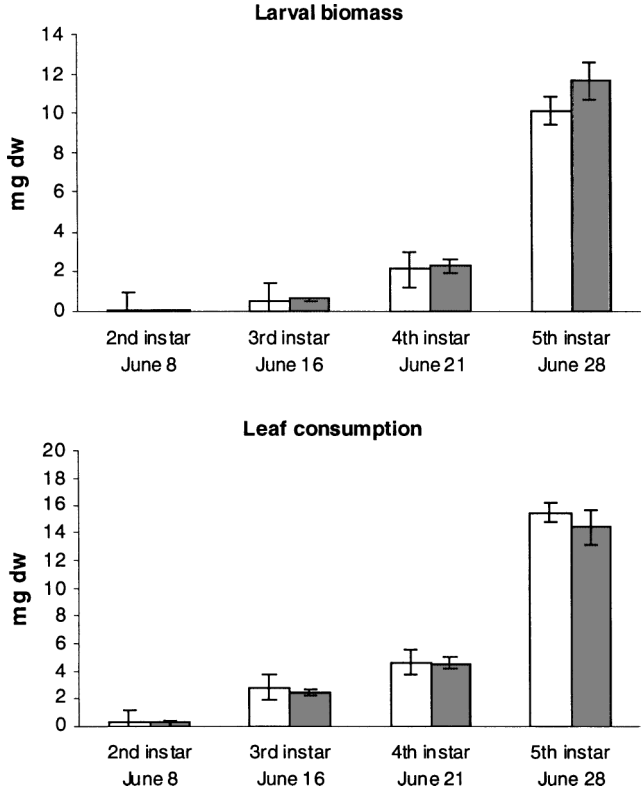

FIG. 4. Leaf consumption by and biomass of *E. autumnata* larvae during early phases of birch leaf development. The clear bars represent controls, and grey bars represent trees with experimentally imposed herbivory. Means and their standard errors are shown.

developed gradually in the course of larval growth, and became significant between the fourth and fifth instars. However, we did not detect effects on pupal mass or leaf consumption, suggesting that *E. autumnata* damage in other parts of the tree may benefit the larvae in terms of growth. This did not increase the biomass losses in mountain birch. Nevertheless, larvae may benefit from a higher growth rate, since large larvae are less susceptible to parasitoids than young ones (Kaitaniemi and Ruohomäki, 1999).

Previous studies of the mountain birch system have reported variable strengths of rapid-induced responses to manual defoliation (Haukioja and Hanhimäki, 1985; Hanhimäki and Senn, 1992). Other studies of birch, also using short pulses of manual defoliation, have demonstrated main effects of defoliation on primary metabolites (protein-bound amino acids, carbohydrates) and on secondary metabolites

TABLE 1. RESULTS FOR REPEATED MEASURES MANCOVA OF *E. autumnata* GROWTH AND LEAF CONSUMPTION

Source of variation	Defoliation	Time	Defoliation[a] time	Leaf phenology
E. autumnata growth	Wilks' lambda = 0.565	Wilks' lambda = 0.029	Wilks' lambda = 0.615	Wilks' lambda = 0.506
	$F_{4,18} = 3.46$, $P = 0.029$	$F_{3,19} = 213.07$, $P < 0.001$	$F_{3,19} = 3.97$, $P = 0.024$	$F_{4,18} = 4.39$, $P = 0.012$
Leaf consumption by *E. autumnata*	Wilks' lambda = 0.888	Wilks' lambda = 0.416	Wilks' lambda = 0.931	Wilks' lambda = 0.578
	$F_{4,18} = 0.57$, $P = 0.69$	$F_{3,19} = 8.90$, $P < 0.001$	$F_{3,19} = 0.47$, $P = 0.71$	$F_{4,18} = 3.28$, $P = 0.034$

Note. Leaf Phenology was used as a covariate.

[a] A defoliation-induced difference in larval mass between the fourth and fifth instar was statistically significant as revealed by analysis of variance contrast (interaction of fourth to fifth instar and treatment, $F_{1,21} = 5.81$, $P = 0.028$).

TABLE 2. PEARSON PRODUCT MOMENT CORRELATION COEFFICIENTS DESCRIBING THE RELATIONSHIPS OF *E. autumnata* PERFORMANCE TRAITS AND BIRCH LEAF PHENOLICS, NUTRITIVE COMPOUNDS, AND WATER[a]

	Initial weight of fifth instar larvae	Leaf consumption by fifth instar larvae	Pupal mass
Total hydrolyzable tannins	0.115	−0.020	0.061
Total galloylglucoses	0.171	−0.113	0.024
Total ellagitannins	0.123	0.009	0.046
Chlorogenic acid	−0.230	0.437*	0.391
Total flavonoids	−0.359	0.551*	0.404
Total proanthocyanidins	−0.107	0.081	−0.062
Total carbohydrates	−0.129	−0.004	0.079
Total protein-bound amino acids	−0.022	0.456*	0.558*
Photosynthesis	0.032	−0.176	0.040
Leaf toughness	−0.201	−0.185	−0.129
Leaf biomass	−0.211	−0.310	0.024

[a] Coefficients with "*" have $P < 0.05$. $N = 24$.

(flavonoids, chlorogenic acid, proanthocyanidins, and hydrolyzable tannins) (Hartley and Lawton, 1987; Keinänen et al., 1999). One possible reason for the discrepancy between these studies and ours may be the type of cue, since most other studies have used heavy manual defoliation, which may produce spurious results with respect to the more natural cues imposed by real herbivores (Karban and Baldwin, 1997). Both artificial and natural defoliation may induce production of systemic wound signals, but the production is more active in natural than in artificial defoliation (Schmelz et al., 2003), and only natural defoliation has a potential for herbivore-specific production of signals, i.e., elicitors (Kessler and Baldwin, 2002).

There were four major differences between our experiment and that of Kaitaniemi and Ruohomäki (2001), which recently reported rapid-induced resistance. First, they used smaller trees and few freely moving larvae to damage trees. Second, they fed larvae with leaves from experimental trees only during bioassays. Third, they found the negative effect in the third instar. Fourth, they point out that free-moving larvae showed the strongest responses to previous herbivory. It is not clear which of the factors are responsible for the difference between our study and Kaitaniemi and Ruohomäki (2001).

We conducted correlational analyses between tree-specific means of putative defensive compounds and tree-specific means of insect performance and consumption to reveal possible mechanisms of plant resistance. Our novel finding is that defoliation modified the relationships between photosynthetic rate and insect consumption. In the control trees, photosynthetic activity and concentrations of monogalloylglucose were positively correlated with leaf consumption. However,

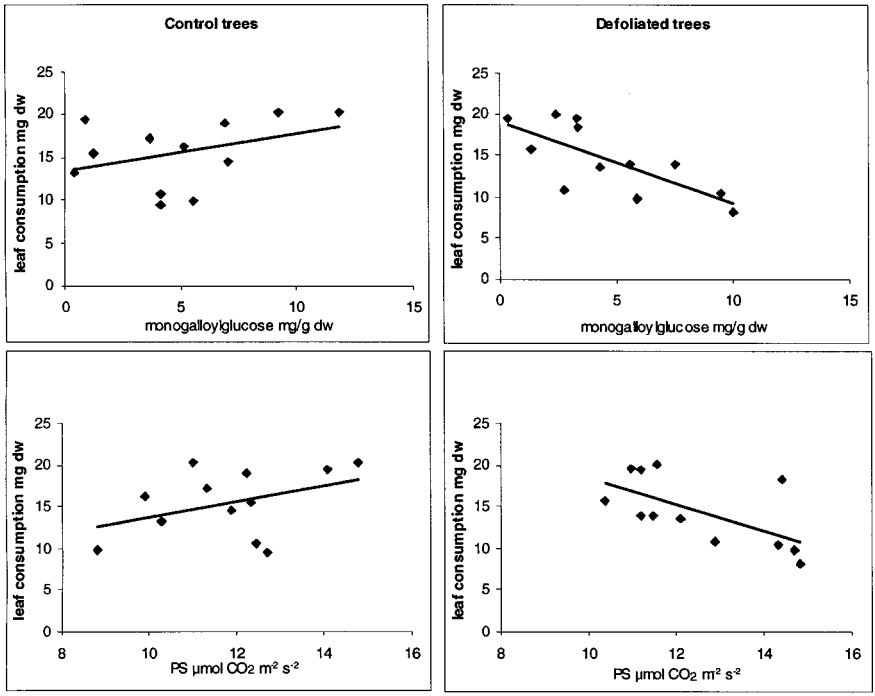

FIG. 5. Scatterplots describing relationships of leaf consumption by fifth instar *E. autumnata* larvae with birch photosynthesis and foliar content of monogalloylglucose in the control and defoliated trees.

in the defoliated trees, photosynthetic activity and monogalloylglucose correlated negatively with leaf consumption. Our results suggest that although *E. autumnata* feeding did not systemically change the levels of measured phenolic compounds, it modified the context in which these compounds act. The effects of monogalloylglucose, and hydrolyzable tannins in general, on herbivore performance are known to range from stimulatory to deterrent (Bernays et al., 1989). Presumably, monogalloylglucose stimulates *E. autumnata* feeding in the absence of previous damage, whereas in defoliated trees the same compound reduces leaf consumption. The physiological effects of hydrolyzable tannins are connected to their fate in the larval digestive tract, i.e., hydrolysis and oxidation, and their ability to precipitate proteins. These processes may have variable effects on herbivore performance (Bi and Felton, 1995; Johnson and Felton, 1996), and damage-induced changes may further modify their effects on herbivores.

The connection between leaf consumption and photosynthetic activity suggests that individual mountain birch trees may meet quite different challenges

during an *E. autumnata* attack. Our data indicate that birch trees with high photosynthetic activity will both encounter lower leaf losses and have higher potential for compensation of biomass losses (by photosynthesis) than trees with low photosynthetic activity. Photosynthetic activity may link herbivore resistance and tolerance in mountain birch because monogalloylglucose is an early product following photosynthesis. The extreme phenotypes with high A_{net} will be able to capture and store more carbon resources during slight defoliation that is typical for early phases of *E. autumnata* outbreaks. This, in turn, may improve their ability to recover after more severe defoliation that is found during the peak phase of *E. autumnata* outbreaks. Extreme trees with low A_{net} may compensate for biomass losses by higher A_{net}, but only partially (Hoogesteger and Karlsson, 1992), indicating that these trees will have a lower capacity to recover. This may partially explain amongsite variation in recovery of birch after the severe defoliation in 1960s (Lehtonen and Heikkinen, 1995).

Our study indicates that only one or two of the measured leaf traits were involved in the rapidly induced responses. However, A_{net} determines the amount of carbon available for plant functions, and monogalloylglucose is the first compound in the pathway of hydrolyzable tannins. The close positive connection between A_{net} and monogalloylglucose suggests plant defense by chemical means may not always be mutually exclusive of plant compensation by growth (Mauricio et al., 1997; Strauss and Agrawal, 1999) contrary to the general assumption (Herms and Mattson, 1992; Haukioja et al., 1998; Koricheva et al., 1998). Rather, the highest concentrations of galloylglucoses are found in the early stages of leaf development (Salminen et al., 2001), and they are part of the hydrolyzable tannin pathway that contributes to cell-wall formation (Grundhoefer and Gross, 2001), indicating a close integration of the hydrolyzable tannin pathway to plant growth.

In summary, limited foliar damage on birch by *E. autumnata* changed the relationships between birch photosynthesis and *E. autumnata* consumption. A similar pattern was found between the most abundant hydrolyzable tannin in birch, monogalloylglucose, and leaf consumption by *E. autumnata*. In contrast, slight defoliation did not change the levels of major primary and secondary metabolites, nor their relationship with larval performance. Slight defoliation of mountain birch that was concentrated in pockets over the whole tree did, however, cause rapidly induced changes that improved larval growth and reduced leaf biomass losses for trees with high photosynthetic activity. The birch preferred by herbivores have the highest potential for successful recovery after herbivore damage.

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TOXIC EFFECTS OF LEMON PEEL CONSTITUENTS ON *Ceratitis capitata*

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Abstract—A series of experiments were conducted to evaluate the toxicity of lemon peel extracts incorporated into mediterranean fruit fly *Ceratitis capitata* diet. Extracts were obtained with different solvents: diethyl ether, ethyl acetate, and methanol. All three extracts were toxic to some extent; the diethyl ether extract was selected for further studies. Ether extracts of lemon peel were prepared weekly over a 2-month period, from fruits collected on the 1st d of the bioassay. Weekly GC-MS and UV analyses of the extracts demonstrated that the concentration of citral and coumarins decreased in the peel after harvest. We conducted a series of bioassays to evaluate the toxicity of the ether extract, and mixtures of this extract with citral, 5,7-dimethoxycoumarin, and linalool incorporated to *C. capitata* larvae's natural diet (lemon slices endocarp) at a concentration of 250 μg/g of diet. Significant larvicidal activity can be obtained from a fresh lemon peel extract; however, when the extract was obtained from stored lemons, toxicity decreased. Addition of small amounts of citral or 5,7-dimethoxycoumarin, and linalool to the stored lemon peel extract would bring back the toxicity to the rates of fresh lemons extracts. Finally, female adults of *C. capitata* fed on diets containing additional amounts of ether extract, 5,7-dimethoxycoumarin, and linalool, were exposed to different photoperiods to test for phototoxicity. The treatment was toxic and affected the oviposition capacity of females depending on photoperiod.

Key Words—*Ceratitis capitata*, lemon peel volatiles, citral, 5,7-dimethoxycoumarin, linalool, toxicity, oviposition inhibition.

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INTRODUCTION

The Mediterranean fruit fly, *Ceratitis capitata*, attacks a wide variety of hosts in subtropical and temperate regions of Argentina, causing serious economic damage and preventing fruit export. Back and Pemberton (1918) and Quayle (1914, 1929) reported that lemons were not attacked by *C. capitata* until the fruit was overripe or partially decayed. These authors attributed the resistance of lemons, among other reasons, to the peel oil. Greany et al. (1983) found that lemon peel oil was toxic to the fruit fly *Anastrepha suspensa* and contributed to making lemons immune from attack. Oxygenated monoterpene aldehydes, like citral, are reported to be responsible for the chemical resistance of lemons to attack by *C. capitata* (da Silva Branco et al., 2000). Additionally, Spitler et al. (1984) considered as extremely low the probability of an infestation of lemons with *C. capitata* in a commercial shipment. Although these reports suggest that lemon is a resistant fruit, systematic research has not been performed to determine which lemon peel compounds are responsible for resistance, or to evaluate the chemical changes in the fruit following harvest (resistance declines significantly after harvest). Identification of toxic lemon peel constituents could be the first step in the investigation of a natural insecticide based on lemon volatiles.

Continuing with our search for natural insecticides (Bardón et al., 1999), we conducted a series of experiments to evaluate the toxicity of lemon peel extracts with different solvents that were incorporated into the insect diet. The ether extract was chosen as an effective insecticide model and its toxicity was evaluated in detail. Finally, on the basis of previous studies (Ashwood-Smith et al., 1983; Nigg et al., 1993) that pointed out the phototoxicity of coumarins to insects, we conducted a second set of experiments to evaluate the effects of different photoperiods on *C. capitata* female adults.

METHODS AND MATERIALS

Chemicals. 5,7-Dimethoxycoumarin was purchased from Aldrich Chemical Company, linalool and citral from Dragoco Chemical Company, and autolyzed brewers yeast from ICN Biomedicals, Inc. All chemicals were used without further purification.

Insects. A colony of several hundred Mediterranean fruit fly adults was initiated with pupae obtained from infested oranges collected from different sites in the Northwest of Argentina. *C. capitata* was reared at Estación Experimental Agroindustrial Obispo Colombres, Tucumán, Argentina, according to the method of Tanaka et al. (1970). Diet consisted of a 3:1 mixture of sugar and yeast hydrolysate suspended in water. Adults were maintained in the laboratory with a photoperiod 12L:12D at 24 $\pm$ 2°C and an RH of 60 $\pm$ 10%. Eggs were

collected during a 1-hr period when females were 7–10-d old (Wong and Nakahara, 1978).

Extraction. Eighty ripe lemons were collected in the middle of the autumn and treated with a 0.3% fungicide (Imazalil) solution by hand spray. Lemons were maintained at 25 ± 2°C and an RH of 60–70% over 2 months. Once a week, five of the collected lemons were selected randomly, and their peels removed from the fruits. Ground peel (200 g) was further extracted with diethyl ether using an ultrasonic bath at 20°C for 20 min. After filtering, the solvent was evaporated to dryness at reduced pressure. The residue was analyzed in triplicate by GC-MS and then bioassayed.

Identification and Quantification of Ether Extract Constituents by GC-MS. Mass spectrometry was carried out by electron impact at 70 eV and 220°C. An HP 6890 Series II chromatograph linked to an HP 5972 mass selective detector with a 30 m × 0.25 mm i.d. HP-5MS 5% phenyl methyl siloxane column was employed. Temperature program: from 50 to 100°C at a rate of 1.5°C/min, from 100 to 160°C at a rate of 3°C/min, and finally from 160 to 280°C at a rate of 10°C/min.

UV Spectroscopy. Coumarins were present in small amounts in citrus peel and detected by UV spectroscopy. Coumarins display strong absorptions in the UV region (λ_{max}-225, 250, 325 nm) and exhibit fluorescence in the visible region of the electromagnetic spectrum. We measured the UV absorption of ethanolic solutions of ether extracts (0.5 mg/ml) weekly at 327 nm with a Shimadzu UV-VIS 160 A spectrophotometer.

Larval Toxicity Bioassay.

Step 1: Portions of 200 g of ground lemon peel were extracted with different solvents (diethyl ether, ethyl acetate, and methanol). After solvent evaporation, an aliquot of each extract (250 μg/g of diet) was added to *C. capitata* diet made of 4 cm diam pulp fruit slices of fresh lemons. They were placed into clear glass Petri dishes (9 cm diam × 1.5 cm tall). Immediately, 20 eggs were placed onto each treated slice and kept at 25 ± 2°C and 60–70% RH for 5 d. On the 5th d of the bioassay, the percentages of hatching and larval mortality were recorded. The experiment was conducted in 10 replicates.

Step 2: The diethyl ether extract containing the volatiles was chosen for further studies on toxicity in relation to chemical composition. Consequently, a second set of bioassays was conducted as follows: weekly, five different treatments (**T1**, **T2**,**T3**,**T4**, and **T5**) were incorporated into *C. capitata* larvae diet (4 cm diam pulp fruit slices of fresh lemons). One of them (**T1**) was the ether extract of lemon peel (obtained with the procedure described above). The extract was dissolved in acetone, and added to slices (15 replicates) at a concentration of 250 μg/g of diet. Slices were placed into clear glass Petri dishes (9 cm diam × 1.5 cm tall) and, after solvent removal, 20 eggs were placed onto each treated slice and kept at 25 ± 2°C and an RH of 60–70% for 5 d. On the 5th d of the bioassay, percentages of hatching and larval mortality were recorded. An identical procedure was followed

with the second treatment (**T2**) that contained 250 μg of ether extract with 1.25 μg of 5,7-dimethoxycoumarin per gram of diet. The third treatment (**T3**) consisted of 250 μg of diethyl ether extract, 1.25 μg of 5,7-dimethoxycoumarin, and 1.25 μg of linalool per gram of diet. The fourth treatment (**T4**) was made of 250 μg of ether extract mixed with 7.5 μg of citral per gram of diet. The fifth treatment (**T5**) consisted of 250 μg of ether extract, 2.5 μg of citral, and 1.25 μg of 5,7-dimethoxycoumarin per gram of diet. Lemon slices treated only with acetone were employed as control after solvent evaporation.

Bioassay of Adult Insects. Three groups of newly emerged *C. capitata* adults were selected from our colony. Each group, consisting of five normal male–female pairs, was placed for 35 d in a small cage to mate and lay eggs. The number of eggs and the mortality of adults were recorded weekly.

The cages were exposed to three different photoperiods; 12L:12D, 6L:18D, and 24 hr (shade), respectively. Adults subjected to treatment fed on a diet containing 100 μg of ether extract, 0.5 μg of 5,7-dimethoxycoumarin, and 0.5 μg of linalool (**T6**) per gram of dry food. Control couples fed on untreated diets. Each experiment had five replicates.

Statistical Analyses. The results are reported as mean $\pm$ SEM. Differences in the mean values were evaluated by analysis of variance (ANOVA). The Tukey test was used for all pairwise multiple comparisons of groups. In all statistical analysis, $P > 0.05$ was not considered significant.

RESULTS AND DISCUSSION

Lemon peel extracts obtained with different solvents contain compounds of different polarities. The methanol and ethyl acetate extracts contain, among others, nonvolatile aromatic compounds like psoralens, coumarins, and flavonoids (Berhow et al., 1998), while the diethyl ether extract contains mainly volatile monoterpenic hydrocarbons (Figure 1), alcohols, and aldehydes Limonene (**1**, CAS# 138-86-3) is the major monoterpene constituent (64–70%).

The diethyl ether extract obtained from fresh lemon peel was the most toxic to mediterranean fruit fly larvae. At a concentration of 250 μg/g of diet, this extract caused 98.8 $\pm$ 3.7% (mean $\pm$ SEM) larval mortality, as shown in Table 1. The diethyl extract was chosen as an insecticide model, and further studies on its chemical composition and bioactivity were performed once a week over a 2-month period with ether extracts obtained from fruits collected on the 1st d of the bioassay. GC-MS analyses indicated that monoterpene alcohols account for approx 1–1.3%, while aldehydes, mainly geranial (**2**, CAS#141-27-5) and neral (**3**, CAS#106-26-3), were 2.5–3% of the ether extract (Marty Klyver et al., 1992, 2000). The natural mixture of the two isomeric aldehydes geranial and neral is known as citral. Rapid

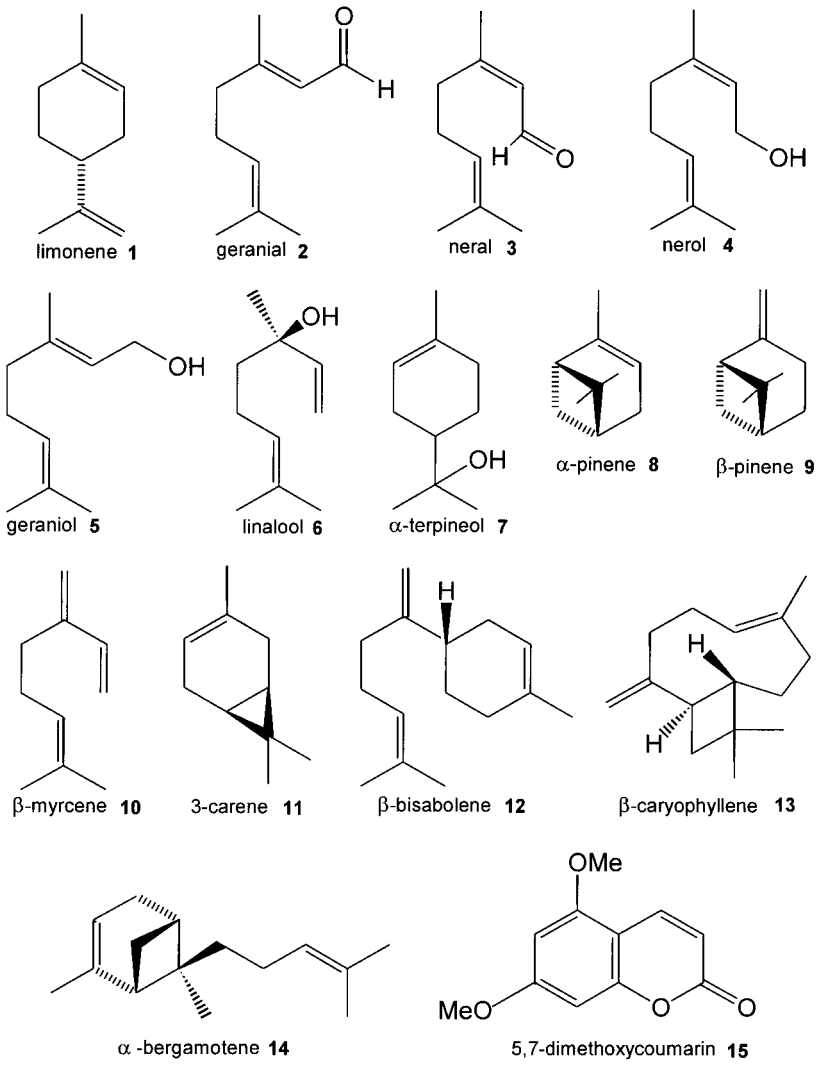

FIG. 1. Ether extract constituents of lemon peel identified by GC-MS.

decay in the concentration of citral (2.6–1.41%) was observed in peels during the 1st month after harvest (Figure 2). The concentration of monoterpenic alcohols (nerol, **4** CAS# 106-25-2; geraniol, **5** CAS# 106-24-1; linalool, **6** CAS# 78-70-6; and α-terpineol, **7** CAS# 98-55-5) was slightly reduced (1.09–0.92%) after

TABLE 1. TOXICITY OF LEMON PEEL EXTRACTS OBTAINED WITH DIFFERENT SOLVENTS AGAINST *C. capitata*

Extracts	% Hatch[a,b]	% Larval Mortality[a,c]
Ethyl acetate	85.5 ± 5.0d	90.9 ± 7.6f
Methanol	85.0 ± 4.1d	94.1 ± 5.5g
Diethyl ether	86.0 ± 3.9d	98.8 ± 3.7h
Control	93.0 ± 5.9e	2.6 ± 3.7i

Note. Means within a column followed by the same letter are not significantly different ($P > 0.05$, Tukey multiple range test).
[a] Numbers in columns represent mean ± SEM; $N = 10$.
[b] Percentage calculated on the basis of number of eggs placed on diets.
[c] Percentage calculated on the basis of number of hatched eggs.

2 months of storage. The concentration of hydrocarbon (limonene, 1 CAS# 138-86-3; α-pinene, **8** CAS# 80-56-8; β-pinene, **9** CAS# 127-91-3; myrcene, **10** CAS# 123-35-3; 3-carene, **11** CAS# 13466-78-9; β-bisabolene, **12** CAS# 495-61-4; β-caryophyllene, **13** CAS# 87-44-5; and α-bergamotene, **14** CAS# 73127-38-5) did not change during the 2 months. Decay in the concentration of total coumarins was detected at λ 327 nm. The concentration decreased 35% over 2 months. The content of 5,7-dimethoxycoumarin (**15** CAS# 487-06-9) was quantitatively determined by GC-MS and found to be reduced by a 59% over 2 months of postharvest storage.

Weekly evaluation of larval toxicity caused by the different treatments is described in Table 2. The diethyl ether extract (**T1**) was responsible for 97.9 ± 2.7% larval mortality compared with the control. Larval mortality decreased significantly to 20.5 ± 6.6% after 2 months ($F = 156.95$; $df = 7, 14$; $P < 0.001$).

Since the coumarin content decreased after harvest, a second treatment, **T2,** was added to the larval diet and was composed of the extract plus an additional amount of the natural lemon peel constituent 5,7-dimethoxycoumarin. The treatment also caused high mortality during 5 weeks, which dropped in the last 2 weeks of the bioassay. In fact, **T2** made with fresh lemon extract caused a 96.2 ± 4.1% decrease in larval mortality, while **T2** prepared with lemon extract obtained 7 weeks after harvest resulted in a 54.6 ± 9.1% decrease in the larval population ($F = 46.79$; $df = 7, 14$; $P < 0.001$).

The third treatment (**T3**) contained the extract, 5,7-dimethoxycoumarin, and the monoterpene alcohol linalool-a potential synergist with insect repellent properties (Assabgui et al., 1997). **T3** affected the larvae during the 2-month bioassay period (Table 2). Mortality increased to 97.2 ± 3.8% with a 2-month-old extract.

Treatments **T4** and **T5** were as toxic to larvae as **T3** during the 2 months of the bioassay. Mortality remained over 90.1% with no significant differences during the 2-month bioassay period.

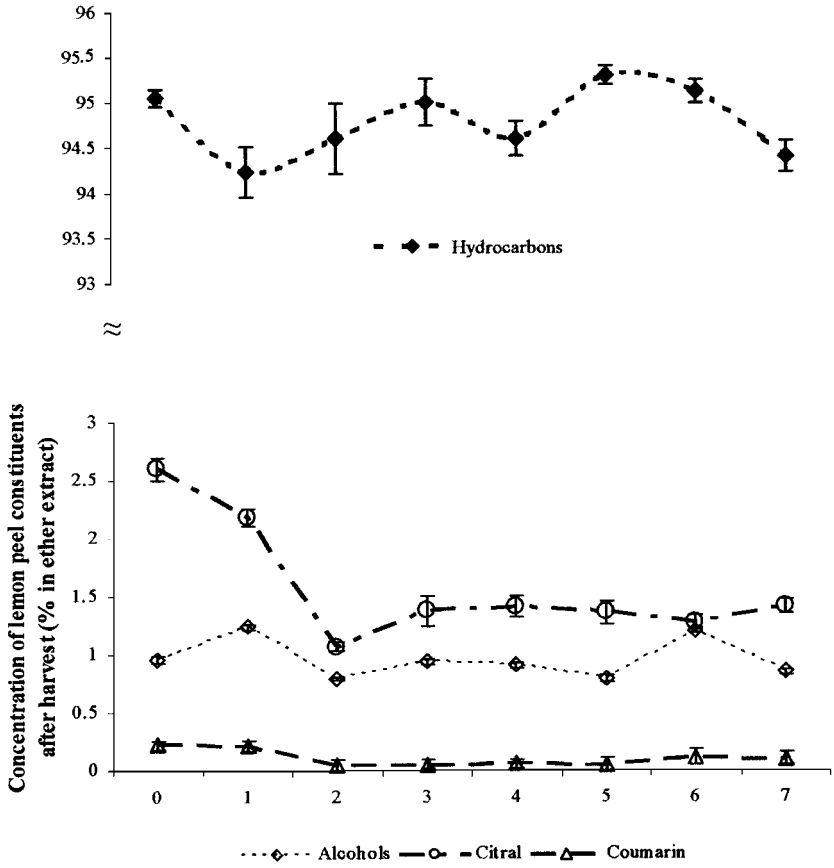

FIG. 2. Variation in the concentration of lemon peel constituents after harvest.

None of the treatments affected the eggs, with no significant differences in the percentage of hatching observed between controls and treatments.

Our results indicate that a potent larvicide can be obtained from fresh. diethyl ether extract of lemon peel. However, if the larvicide is obtained from stored lemons, only the addition of small amounts of citral (**T4**) or citral and 5,7-dimethoxycoumarin (**T5**) can bring the toxicity of the extract back to the levels of fresh lemons. As shown in Table 2, the association of the diethyl ether extract with small amounts of 5,7-dimethoxycoumarin and linalool (**T3**) has the larvicidal effects of the extract of fresh lemon, even when the extract is from stored lemons.

The coumarin 5,7-dimethoxycoumarin induces mutagenesis in bacteria (Ashwood-Smith et al., 1983) as well as being a phototoxic (Nigg et al., 1993). Because of the phototoxic nature of this coumarin, we evaluated the effects of our

TABLE 2. EFFECTS OF TREATMENTS **(T1, T2, T3, T4** AND **T5)** INCORPORATED IN LARVAL DIET OF *C. capitata*[a]

Weeks after harvest	Ether extract **(T1)** (% mortality)[b]	Ether extract + coumarin **(T2)** (% mortality)[b]	Ether extract + coumarin + linalool **(T3)** (% mortality)[b]	Ether extract + citral **(T4)** (% mortality)[b]	Ether extract + coumarin + citral **(T5)** (% mortality)[b]	Control (% mortality)[b]
0	97.9 ± 2.7cA	96.2 ± 4.1cA	98.9 ± 2.2cA	95.3 ± 4.4cA	98.2 ± 2.5cA	3.3 ± 3.9i
1	95.8 ± 5.3cA	93.6 ± 6.8cA	97.5 ± 3.4cA	93.5 ± 5.4cA	96.5 ± 2.6cA	2.4 ± 2.9i
2	79.9 ± 10.4dB	93.0 ± 4.5cA	98.2 ± 3.9cA	93.1 ± 3.8cA	98.2 ± 2.5cA	2.8 ± 3.3i
3	63.9 ± 10.0eD	88.5 ± 7.5cC	98.2 ± 2.6cC	92.3 ± 7.1cA	98.5 ± 2.4cA	2.3 ± 3.4i
4	66.7 ± 8.6eC	78.6 ± 9.0dB	98.9 ± 2.2cA	93.8 ± 5.6cA	97.5 ± 2.3cA	2.7 ± 3.1i
5	50.9 ± 12.4fC	77.8 ± 8.2dB	98.6 ± 3.1cA	91.4 ± 7.2cA	97.2 ± 3.2cA	3.1 ± 2.8i
6	36.2 ± 8.7gC	68.6 ± 8.7eB	98.5 ± 3.2cA	92.9 ± 4.8cA	96.1 ± 5.1cA	3.1 ± 3.6i
7	20.5 ± 6.6hC	54.6 ± 9.1fB	97.2 ± 3.8cA	89.9 ± 5.0cA	95.6 ± 4.3cA	2.7 ± 3.2i

[a] Numbers in columns represent mean ± SEM; $N = 15$.

[b] Means within a column or row followed by the same letter (lower and upper case, respectively) are not significantly different ($P > 0.05$, Tukey multiple range test).

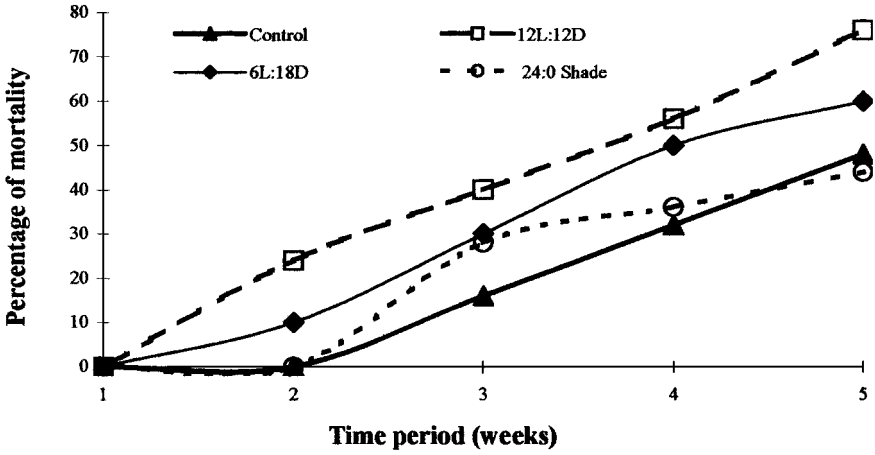

FIG. 3. Mortality of *C. capitata* adult females that fed on treated diets and were exposed to three photoperiods, 12L:12D, 6L:18D, and 24:0 (shade).

treatment (100 μg of ether extract + 0.5 μg 5,7-dimethoxycoumarin + 0.5 μg linalool per gram of diet) on adults of *C. capitata* under three different light regimes (Figure 3). Females exposed to a 12L:12D photoperiod were significantly affected by treatment ($F = 16.05; df = 3, 24; P < 0.001$). Mortality was not observed in the control during the first 2 weeks, while the mortality rose to 25% in the 12L:12D experiment. After 5 weeks, the number of dead females doubled that of the control. In addition, as shown in Figure 4, the oviposition

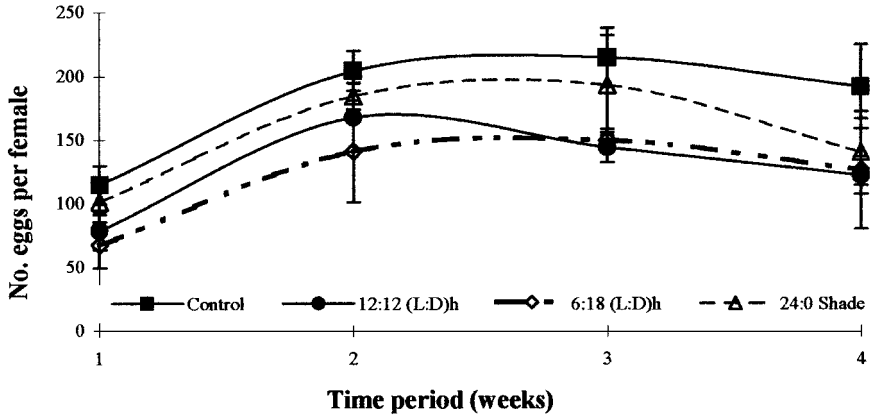

FIG. 4. Number of eggs laid by females that fed on treated diets and were exposed to three photoperiods 12L:12D, 6L:18D, and 24:0 (shade).

capacity of females in the 12L:12D photoperiod conditions was significantly altered ($F = 11.72$; $df = 3, 19$; $P < 0.001$). The number of eggs laid by the females exposed to a 12L:12D photoperiod was half the number of the control, indicating that a longer exposure to light affects oviposition capacity to a greater extent.

Our results indicate that a mixture of nonpolar constituents of lemon peel with additional amounts of citral, 5,7-dimethoxycoumarin, and linalool might be useful as a natural insecticide for treatment of larvae and adults at the concentrations tested. However, because no differences were observed in the alimentary behavior of the adults toward treated and control diets, the treatment might be added to bait with good results as a bait–pesticide mixture.

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HOST-PLANT SPECIALIZATION IN PHEROMONE STRAINS OF THE EUROPEAN CORN BORER *Ostrinia nubilalis* IN FRANCE

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Abstract—European corn borer (ECB) feeding on maize (*Zea mais*), mugwort *(Artemisia vulgaris*), and hop (*Humulus lupulus*) are genetically different in France and referred to as host-plant races. Here, we investigated sex pheromone composition as a possible trait linked to the host plant. ECB host races were sampled from 13 different sites in France. GC-MS analysis of female pheromone showed that 175 out of 176 maize females belonged to the Z type with one hybrid. In contrast, mugwort and hop females belonged almost exclusively to the E type. No Z females were found on these plants and only 2 females out of 169 were hybrids. In the three sites of sympatry, the hybrid proportion was far from Hardy–Weinberg expectations. Wind tunnel experiments showed that 76–79% of maize males from three populations were attracted by Z females, whereas neither mugwort nor hop males were. Mugwort males from Toussus-le-Noble were attracted by E females originating from an American maize strain. These data showed that maize, mugwort, and hop host races of *O. nubilalis* differ not only in their host plant but also in the sex pheromone they use. Because mugwort and hop are putative ancestral host plants, these results are discussed from the point of view of evolutionary scenarios for the emergence of Z and E strains.

Key Words—Pheromone strain, host race, sympatric speciation, reproductive isolation.

INTRODUCTION

The pheromone-mediated mate location system in European corn borer (ECB) has been investigated extensively since the identification of female-produced

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(*Z*)-11-tetradecenyl acetate (Z11-14:OAc) as a sex attractant by Klun and Brindley (1970). Early studies (Klun et al., 1973; Kochansky et al., 1975) revealed a polymorphic pheromonal system with respect to the Z11-14:OAc/E11-14:OAc ratio. In the Z strain, females release Z11-14:OAc and E11-14:OAc in a 97/3 ratio, and males respond specifically to this blend (Glover et al., 1987). In turn, the E strain is characterized by a blend ranging from 1/99 to 4/96 of Z11-14:OAc/E11-14:OAc, respectively (Klun and Cooperators, 1975; Anglade et al., 1984; Peña et al., 1988). Major differences in pheromone production, male perception, and male behavioral response are genetically controlled (Klun and Maini, 1979; Roelofs et al., 1987; Löfstedt et al., 1989; Glover et al., 1991). Consequently, considerable emphasis has been placed on *Ostrinia nubilalis* (Hübner) and related species to gain a better understanding of moth pheromone evolution and of the speciation process involved. Z and E strains have been found in *O. nubilalis* and *O. scapulalis* (Walker) (Huang et al., 1997, 2002) but their taxonomic status is controversial (Cardé and Roelofs, 1978; Linn and Roelofs, 1995). Buttlin (1995) stressed that "their origin and fate are still an open question." These pheromone strains are regarded as an example of an in-progress or a recent sympatric or parapatric speciation process among Lepidoptera because of strong assortative mating. Different evolutionary scenarios were proposed to explain the emergence and persistence of such a polymorphic system, despite evidence of interbreeding in the laboratory and the field (Löfstedt, 1990, 1993; Buttlin and Trickett, 1997; Ishikawa, 1999). Furthermore, only maize (*Zea mais* L.) infesting populations have been investigated until recently, and occurrence of Z and E strains on other host plants has not yet been studied. Recent studies have focused on gene exchange among ECB populations feeding on maize, mugwort (*Artemisia vulgaris* L.), and hop (*Humulus lupulus* L.), the latter being putative ancestral host plants, whereas maize was only recently introduced into Europe (around the sixteenth century). Populations feeding on these different host plants were shown to be genetically differentiated with allozymic and mitochondrial marker frequencies (Bourguet et al., 2000; Martel et al., 2003) and were, thus, referred to as host-plant races. Because the pheromone specific mate recognition system might be linked to this genetic differentiation (Thomas et al., 2003), we investigated ECB sex pheromone in populations feeding either on *Z. mais*, *A. vulgaris*, or *H. lupulus*. Pheromone from individual females was collected by solid phase micro-extraction (SPME) and analyzed using GC-MS. Furthermore, cross-attraction between individuals of the three different host races and Z or E laboratory strains was studied in a wind tunnel.

MATERIALS AND METHODS

Insects

Sites. Sixteen samples of ECB populations characterized by their host plants and collection locations were collected between 1999 and 2002. In three locations,

TABLE 1. CHARACTERISTICS OF *Ostrinia nubilalis* SAMPLES COLLECTED ON MAIZE, MUGWORT, AND HOP IN VARIOUS LOCATIONS OF FRANCE

Host plant	Location (department)	Latitude	Longitude	Collected instars	Collection date month/year
Maize Mugwort	Plaisir (Yvelines)	48°49′N	1°57′E	Diapausing larvae	Sept. 2000 April 2002
Maize Mugwort	Darvoy (Loiret)	47°52′N	2°06′E	Diapausing larvae	May 2001 April 2002
Maize Hop	Bourg Saint Andéol (Ardèche)	44°22′N	4°39′E	Larvae	Sept. 2001
Maize	Vancia (Rhône)	45°50′N	4°55′E	Larvae	July 2000
	Pierrelatte (Drôme)	44°23′N	4°42′E	Larvae	August 2000 April 2002
	Revel (Haute Garonne)	43°28′N	2°00′E	Diapausing larvae	June 2001
	Villasavary (Aude)	43°13′N	2°02′N	Eggs (51 egg masses)	June 2001
Mugwort	Vermelle (Pas-de-Calais)	50°29′N	2°44′E	Larva	June 2001
	Toussus-le-Noble (Yvelines)	48°45′N	2°07′E	Diapausing larvae	April 2000
Hop	Laventie (Pas-de-Calais)	50°38′N	2°46′E	Diapausing larvae	June 2000
	Obernai (Bas-Rhin)	48°28′N	7°29′E	Diapausing larvae	Sept. 1999
	Saint Marcel d'Ardèche (Ardèche)	44°19′N	4°37′E	One larva and eggs from feral adults (10♂ + 10♀)	August 1999
	La Cadiere-d'Azur (Var)	43°12′N	5°46′E	17 adults	May 2000

Darvoy, Plaisir, and Bourg-Saint-Andéol, ECB were collected both on maize and neighboring wild host plants such as mugwort or hop (see Table 1 and Figure 1). In 10 other sites, ECB were collected on a single species of host plant, i.e., either maize (four sites), mugwort (two sites), or hops (four sites).

Collection. O. nubilalis (Lepidoptera: Crambidae) were collected from host plants at different development stages: diapausing or nondiapausing larvae, egg masses, and in two cases as adults (Table 1). At Saint Marcel d'Ardèche, only one larva was found in a hop stem but unoccupied tunnels, parasitoid-killed larvae, and empty pupae indicated that *O. nubilalis* had infested the hop plants. In this

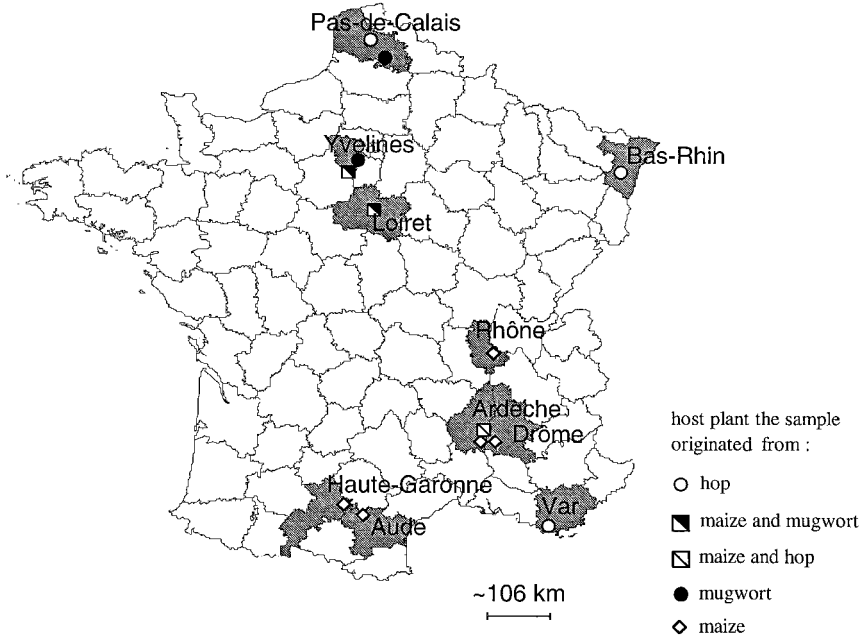

FIG. 1. Collection sites of European corn borer for pheromone analysis and wind-tunnel experiments. French department/locations: Yvelines/Plaisir, Toussus-le-Noble, Loiret/Darvoy, Ardèche/Bourg Saint Andéol, Saint Marcel d'Ardèche, Rhône/Vancia, Drôme/Pierrelatte, Haute–Garonne/Revel, Aude/Villasavary, Pas-de-Calais/Vermelle, Laventie, Bas-rhin/Obernai, Var/La Cadière d'Azur.

location, 10 males and 10 females were caught as adults and used to establish a laboratory colony for two generations. The colony from La Cadière d'Azur was established from 17 adults caught flying around wild hop plants. In both cases, the adults were placed together in a mating cage, and eggs obtained were used to set up colonies. As neither maize nor *A. vulgaris* plants were present in the vicinity of hop plants in both locations, we considered that all the adults caught originated from hop plants.

Eggs and nondiapausing larvae sampled on host plants were also reared on artificial diet under the following conditions: 16:8 hr L:D photoperiod, $T = 24 \pm 2^{\circ}$C, R.H. $= 60 \pm 10\%$. Diapausing larvae collected before winter were kept 2 months at 4°C, under a short day photoperiod (8:16 hr L:D) and then 1 month at 15°C and a 8:16 h L:D photoperiod before placing them under rearing room conditions until adult emergence. Diapausing larvae collected between March and June were directly placed under long day photoperiod (16:8 hr L:D, $T = 24 \pm 2^{\circ}$C, R.H. $= 60 \pm 10\%$). Water was provided to adults on a soaked cotton pad.

Pheromone Analysis

Insects. Sexes were separated as pupae, and newly emerged females were kept individually in plastic containers, with water supplied, under the conditions described above. Two to five d-old females were used for sex pheromone collection within the last 3 hr of scotophase, during the calling period. Pheromone phenotype frequencies of Saint Marcel d'Ardèche and La Cadière d'Azur could have been biased by pooling together, for each location, the eggs laid by adults caught in the field. The actual number of females that oviposited was not determined, and sibling individuals could have been analyzed. However, the number of adults used reduced this possibility.

Pheromone Collection. Pheromones were collected by SPME as described by Frérot et al. (1997). The pheromone gland was extruded by gentle pressure on the abdomen and kept in this position with metallic forceps. A Supelco SPME fiber (65 μm Carbowax^{TM}-Divinylbenzene) previously cleaned by thermal desorption (5 min in the GC injector at 240°C) was gently rubbed on the gland for 4 min at room temperature. Care was taken to avoid contact with scales and anal droplets. Each fiber was then either directly analyzed or wrapped in aluminium foil and stored at −20°C until analysis.

Pheromone Identification. The components of the sex pheromone were identified using a Varian 3400 gas chromatograph (GC) linked to a Saturn 2 mass spectrometer (electron impact mode, 70 eV, 40–300 amu). The GC was equipped with a split–splitless injector and a MDN5 S column (SUPELCO; 30 m × 0.32 mm ID, 0.5 μm film). The components adsorbed onto the SPME fiber were thermally desorbed for 2 min in the injector at 250°C. The oven temperature was programmed from 50 to 300°C at 8°C min^{-1}; helium (11 psi) was the carrier gas. Compounds were indentified by comparison of retention times and mass spectra of the natural compounds with those of synthetic reference samples.

The Z11-14:OAc/E11-14:OAc ratio was calculated to infer pheromone phenotype. Individuals were classified as Z when Z11-14:OAc percentage ranged from 100 to 85%; E when Z11-14:OAc percentage ranged from 0 to 15%, and hybrid when Z11-14:OAc percentage ranged from 20 to 50%. To keep the ratio of Z11-14:OAc and E11-14:OAc comparable whatever the number of minor components, the sum of these two components was arbitrarily set as 100. The ratio of the minor components was expressed relative to the sum of Z11-14:OAc and E11-14:OAc. For example, a female with 10 ng Z11-14:OAc, 0.3 ng E11-14:OAc, 2.25 ng Z11-16:OAc, and 0.5 ng 14:OAc is presented as (Z11-14:OAc/E11-14:OAc)/Z11-16:OAc/14:OAc ratio of (97/3)/22/5.

Wind-Tunnel Experiment

As the response of ECB males stimulated by a pheromone source is ratio specific (Linn et al., 1985; Glover et al., 1987), wind tunnel bioassays with females

belonging to an identified pheromone strain were used to determine the male pheromone status. Reciprocally, tests with already known Z or E males allowed us to determine the female pheromone strain. Furthermore, interpopulation attraction provided information on the strength of pheromone driven premating reproductive isolation.

Wind Tunnel. The wind tunnel was hemicylindrical (150 cm in length, 80 cm in width, and 45 cm in height). All tests were carried out during the last 3 hr of scotophase at 20°C and relative humidity 60 ± 10%. The wind speed during all experiments was 0.25 m s^{-1} and light <2 lux. Red light allowed us to observe the insects directly.

Females as Pheromone Source. The pheromone gland of 2 to 5-d-old calling females was artificially kept everted with metallic forceps. Each female was used to test five to six individual males. Previous observations have shown that artificially everted glands remain attractive even longer than the time necessary for testing six males. Two reference strains were used to test cross-attraction: a Z strain reared at INRA Le Magneraud (referred to as Z INRA) and an E strain (referred to as E Cornell) provided by C. Linn (Cornell University). Both reference strains were laboratory colonies originating from individuals collected on maize. The composition of their pheromone blend was previously verified by GC-MS.

Wind Tunnel Protocol. Naive males were introduced individually in a 5.5 cm diam. × 8 cm wire mesh cylinder, placed on a 10-cm high platform with the open end of the cylinder upwind, 120-cm downwind of the source in the pheromone plume. Each insect was allowed 2 min to initiate flight. Males that did not take flight after 2 min were considered as no-responding males. For each male taking off, the behavioral response was observed to determine the percentage of males reaching the source. This was defined as touching and/or landing on the pheromone source, most of the time with courtship displays (i.e., clasper extrusion, abdomen movement, wing fanning). The forceps and glass pipette holding the female were washed with ethanol when renewing the female. Males from six populations were tested: three populations from maize (Darvoy, Pierrelatte, and Vancia), one from mugwort (Toussus-le-Noble), and two from hop (Saint Marcel d'Ardèche and Laventie). The response of Z INRA male was investigated with females from two maize populations (Darvoy and Vancia) and from two hop populations (Saint Marcel d'Ardèche and Laventie). Cross-tests between mugwort males and females from the American E Cornell strain were also carried out.

RESULTS

Pheromone Analysis

Maize Race. Pheromone components were not detected from 41 out of 217 female gland anlayses. The remaining 176 glands produced enough pheromone

TABLE 2. DISTRIBUTION OF PHEROMONE PHENOTYPES RELATED TO HOST PLANT AND GEOGRAPHICAL LOCATION

			Pheromone phenotype		
Host plant	Location	*N*	Z	Hybrid	E
Maize	Plaisir	41	41	0	0
	Darvoy	39	39	0	0
	Vancia	10	10	0	0
	Pierrelatte	42	42	0	0
	Bourg Saint Andéol	9	9	0	0
	Villasavary	30	29	1	0
	Revel	5	5	0	0
Total (%)		176	175 (99.4%)	1 (0.6%)	0
Mugwort	Plaisir	81	0	1	80
	Darvoy	20	0	0	20
	Toussus-le-Noble	19	0	0	19
	Vermelle	5	0	0	5
Total (%)		125	0	1 (0.8%)	124 (99.2%)
Hop	Laventie	3	0	0	3
	Obernai	10	0	0	10
	Bourg Saint Andéol	13	0	1	12
	Saint Marcel d'Ardèche	9	0	0	9
	La Cadiere-d'Azur	9	0	0	9
Total (%)		44	0	1 (2%)	43 (98%)

Note. Pheromone phenotypes were identified by GC-MS and classified as Z when the Z11-14:OAc percent in the E11-14:OAc/Z11-14:OAc blend was higher than 85%, hybrid when the Z percent ranged from 20 to 50%, and E when this percent was below 15%.

to obtain reliable GC traces and mass spectra (Table 2). Z11-14:OAc was the main stereoisomer in the blend of 175 females (99.4%) that clearly belonged to the Z pheromone strain. Among Z females, E11-14:OAc was detected as a minor component in 66 glands at an average Z:E ratio of 97.7/2.3 $\pm$ 6.3 (mean $\pm$ SD). For the other 109 glands, E11-14:OAc was not detected (detection limit around 100 pg). A single female originating from Villasavary exhibited a hybrid pheromone blend with a ratio of 49.8/50.2 Z11-14:OAc/E11-14:OAc. No E females were identified. The saturated component 14:OAc was detected in 61 female glands at a ratio of (Z11-14:OAc/E11-14:OAc)/14:OAc (97.7/2.3)/17.1 $\pm$ 10.0 (Table 3). Females producing 14:OAc did not originate from a single site of collection but were found in three localities: Darvoy, Plaisir, and Revel. Z11-16:OAc was present in five female glands of the Z type, with a (Z11-14:OAc/E11-14:OAc)/Z11-16:OAc ratio of (97.7/2.3)/7.5$\pm$ 7.0. These five females originated from different sites of

TABLE 3. PHEROMONE BLEND OF THREE *Ostrinia nubilalis* HOST RACES IDENTIFIED BY SPME COLLECTION AND GC-MS ANALYSIS OF INDIVIDUAL FEMALE GLANDS

Host plant	Z11-14:OAc	E11-14:OAc	Z11-16:OAc	14:OAc	*N*
Maize (*Z. mais*)	97.7 ± 6.3	2.3 ± 6.3	7.5 ± 7.0	17.1 ± 10.0	175
	$n = 66$	$n = 66$	$n = 5$	$n = 61$	
Mugwort (*A. vulgaris*)	1.1 ± 1.3	98.9 ± 1.3	28.5 ± 23.6	4.6 ± 4.8	123
	$n = 7$	$n = 7$	$n = 109$	$n = 86$	
Hop (*H. lupulus*)	0 ± 0.0	100 ± 0.0	19.1 ± 17.3	5.1 ± 2.8	43
	$n = 43$	$n = 43$	$n = 35$	$n = 31$	

Note. N is the number of Z or E individuals identified. *n* is the number of females producing the particular minor component out of *N*. One mugwort female with a Z11-14:OAc/E11-14:OAc/Z11-16:OAc ratio of 0/100/961 and three hybrid individuals were discarded for ratio calculation.

collection (Darvoy, Vancia, Pierrelatte, Villasavary). Z11-16:OAc was absent from the gland of the single hybrid individual.

Mugwort Race. One hundred and twenty-six of the one hundred and thirty-nine mugwort females analyzed contained sufficient pheromone to identify E11-14:OAc as the main component. One hundred and twenty-four were E (Table 2). A single individual remained undetermined. The low amount of E11-14:OAc detected did not allow us to reject the possibility of a hybrid individual: in a E/Z ratio 65/35, Z11-14:OAc would be represented by a quantity below the detection threshold. No Z female was identified in the mugwort race, but one hybrid was found at the Plaisir site. The gland of this hybrid contained Z11-14:OAc/E11-14:OAc in a ratio 45/55. Traces of the Z11-14:OAc isomer were detected in seven E females at a mean percentage of Z11-14:OAc/E11-14:OAc $1.1 \pm 1.3/98.9$. Five of these females with traces of Z11-14:OAc belonged to the larger sample (Plaisir), and two of them belonged to the Darvoy sample. The GC procedure made detection of Z11-14:OAc traces more difficult than E isomer detection because of the presence of a nonpheromonal compound eluting close to the Z11-14:OAc peak. Out of 124 E females, Z11-16:OAc and 14:OAc were identified in 110 and 87 female glands, respectively. Females with Z11-16:OAc and 14:OAc were equally represented in all mugwort population samples. In individuals producing low amounts of the main component, the expected amount of Z11-16:OAc and 14:OAc were below the detection threshold, according to the observed ratio. Thus, it cannot be determind whether these individuals did not produce minor components or whether minor components were not detected. In turn, when the amounts of E11-14:OAc were high, Z11-16:OAc and 14:OAc were present. Thus, we considered that they were part of the pheromone blend. After elimination of one outlier individual releasing a ratio of (Z11-14:OAc/E11-14:OAc)/Z11-16:OAc/14:OAc (0/100)/961/13.7, the average pheromone blend for the remaining females was $(1.1/98.9)/28.5 \pm 23.6/4.6 \pm 4.8$ (Table 3).

Hop Race. Forty-four out of the forty-seven female glands analyzed produced enough pheromone for pheromone-type identification. Forty-three females belonged to the E strain, and one individual found in Bourg Saint Andéol yielded a hybrid blend with Z11-14:OAc/E11-14:OAc in a ratio of 45.9/54.1. In most E females (81%) and in the hybrid individual, Z11-16:OAc was identified, respectively, in a (Z11-14:OAc/E11-14:OAc)/Z11-16:OAc ratio of (0/100)/19.1 $\pm$ 17.3 (Table 3) and (45.9/54.1)/6.3 for the single hybrid. Seventy-two percent of the E females also produced 14:OAc in (Z11-14:OAc/E11-14:OAc)/14:OAc ratio of (0/100)/5.1 $\pm$2.8. This component was not detected in the hybrid female gland. Hop females released a blend belonging to the E type with the additional components Z11-16:OAc and 14:OAc. The (Z11-14:OAc/E11-14:OAc)/Z11-16:OAc/14:OAc ratio in hop and mugwort females did not differ. At the three locations where ECB were sampled on maize and mugwort or hop growing in sympatry, the observed frequencies of pheromone phenotypes were different from the expected distribution under Hardy–Weinberg equilibrium (Table 4). In all cases, the proportion of hybrids was lower than expected.

Wind Tunnel. In control tests, about 76% of Z INRA males submitted to effluvia of a Z INRA female reached the source (Figure 2). All interpopulation tests performed between Z INRA males and females collected on maize showed a high level of cross-attraction. Z INRA females attracted most of the males collected on maize from Darvoy (82%), Pierrelatte (79%), and Vancia (76%) (Figure 2A). Maize females from Darvoy and Vancia attracted, respectively, 61–74% of Z INRA males. In contrast, cross-attraction did not occur between mugwort or hop individuals and the Z INRA strain. Out of 35 mugwort males and 41 hop males, few took off (20 and 7%, respectively), and none reached the Z INRA females. Reciprocally, Z INRA males did not respond to hop females (Figure 2B). No contact with the female was observed, and the percentage of males taking off was 4% with Z INRA males when confronted with Saint Marcel d'Ardèche and Laventie

TABLE 4. COMPARISON OF PHENOTYPE FREQUENCIES OBSERVED AT THREE SITES OF SYMPATRY WITH EXPECTED FREQUENCIES UNDER HARDY-WEINBERG EQUILIBRIUM

	Pheromone phenotype		
Sites	Z	Hybrid	E
Darvoy	39 (26[a])	0 (26[a])	20 (7[a])
Plaisir	41 (14[a])	1 (55[a])	80 (53[a])
Bourg Saint Andéol	9 (4[a])	1 (11[a])	12 (7[a])

[a] The distribution observed is different from the theoretical distribution calculated under Hardy–Weinberg equilibrium hypothesis (chi-square test, df = 2, $\alpha = 0.01$).

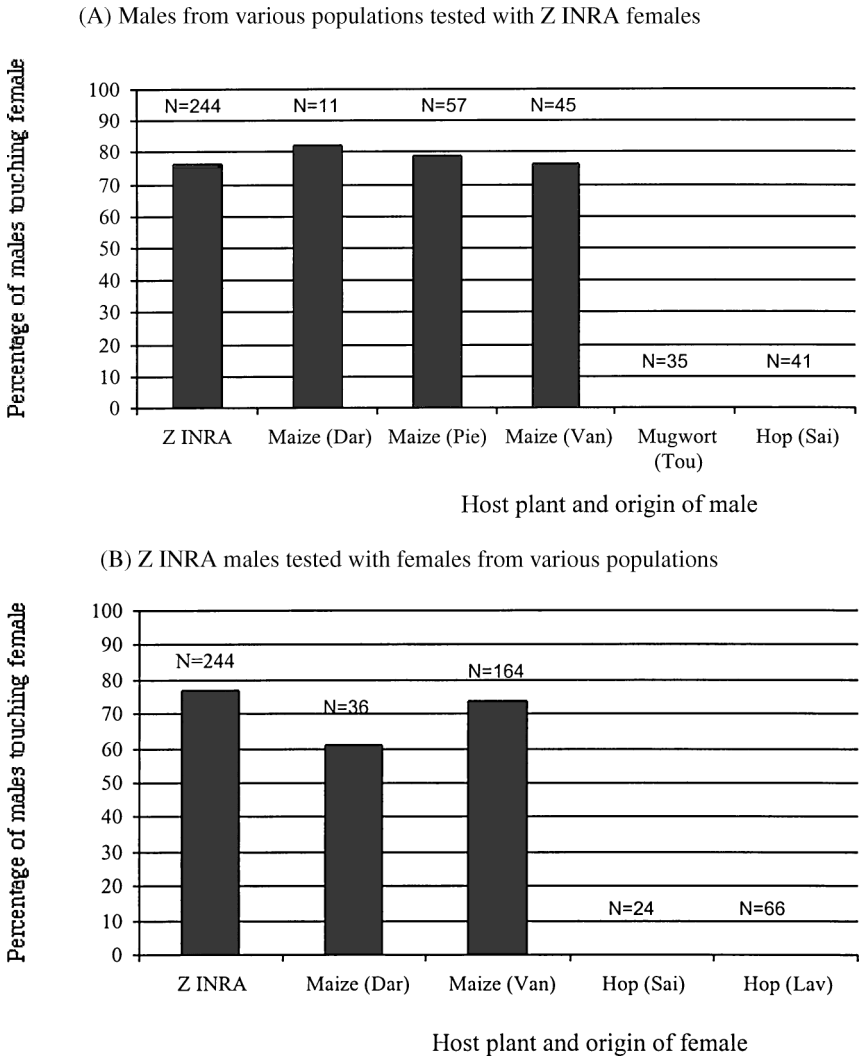

FIG. 2. Cross-test in a wind tunnel between various host-race populations and the reference Z INRA population. (A) Males from various populations tested with Z INRA females. (B) Z INRA males tested with females from various populations. Dar: Darvoy, Pie: Pierrelatte, Sai: Saint Marcel d'Ardèche, Tou: Toussus-Le-Noble, Lav: Laventie, Van: Vancia. Distance between male and source in the wind tunnel is 1.2 m.

females originating from hop. Those behavioral results confirmed data obtained from female pheromone gland analysis: the Z pheromone type was widely distributed among maize populations whereas mugwort and hop populations were almost exclusively the E type. Cross-attention tests with mugwort individuals from Toussus-le-Noble and Z INRA or E Cornell populations originating from maize, showed that mugwort males ($N = 35$) did not respond to Z INRA females, whereas 87% ($N = 35$) were attracted by E Cornell females.

DISCUSSION

In this study, the pheromone-mediated mate location systems of maize, mugwort, and hop ECB races were investigated to determine the mechanism of reproductive isolation between host races of *O. nubilalis.* Sex pheromone was evaluated as a putative factor involved in genetic differentiation previously described between the maize race, and the mugwort and hop races (Bourguet et al., 2000; Martel et al., 2003; Thomas et al., 2003). This study is the first investigation of *O. nubilalis* sex pheromone in correlation with introduced host plant (maize) and native host plants (mugwort and hop). A clear link between host-plant exploitation and pheromone phenotype was shown in the area studied. The E phenotype was found only in mugwort and hop ECB populations, whereas the Z phenotype was identified only in maize populations. On each host plant, ECB individuals expressing the hybrid phenotype were rare (<1%).

The localization of the collection sites in the different parts of France and the specific study of three sites of sympatry demonstrated that the association of Z and E strains with a specific host plant was not due to differences in geographical distribution. In Europe, former studies located the E strain only in northern France (Anglade et al., 1984), southern parts of Switzerland (Buechi et al., 1982; Anglade et al., 1984), Italy (Anglade et al., 1984; Peña et al., 1988), Hungary, and Poland (Anglade et al., 1984). Here, the occurrence of the E strain in southern France was reported for the first time. When hop was available in southern France, the plant was infested by the E strain of *O. nubilalis*, even if sympatric maize was found to be exclusively infested by the Z strain.

The strong link between host plant and pheromone phenotype may involve (i) differences in moth emergence timing related to host-plant phenology, (ii) differential mortality of each pheromone strain on the three host plants and/or (iii) female host plant choice. In the Ile-de-France region, where ECB has only one flight, Thomas et al. (2003) have shown that E mugwort individuals emerge sooner than Z maize individuals. This biological trait should locally favor colonization of mugwort patches by E mugwort individuals because mugwort develops before maize is planted in this area (Béthenod and Bourguet, personal communication). However, such a process is unlikely to play a key role in places where ECB are polyvoltine

and can develop on different phenological stages of their host plants. No information was available concerning voltinism of *O. nubilalis* feeding on hop in the Southeast of France. However, personal observations of ECB moths flying in hop fields in mid-August and the presence of empty pupae in young hop stems in early September suggested that those populations were bivoltine. Thus, southern populations of the E strain have the same number of generations per year as Z populations observed on neighboring maize. The differential mortality hypothesis is unlikely because E individuals are described on maize in other countries, and Z individuals can complete development in mugwort stalks (Frérot, unpublished data). Thus, female choice for oviposition sites might be a key factor. Inherited or learned patterns of host preference for ovipositing females are often involved in insect host races or biotype differentiation (Diehl and Bush, 1984; Renwick, 1994), and our data suggest that oviposition behavior of Z and E strains should be investigated.

The host-plant segregation of ECB pheromone strains described in this study contrasts with former reports of the presence of E strain in maize fields in other areas (Buechi et al., 1982; Anglade et al., 1984; Roelofs et al., 1985; Klun and Huettel, 1988; Peña et al., 1988). Because some of those earlier studies relied totally or partially on field trapping, they have to be considered with caution since E males might have flown from mugwort or hop growing in the vicinity of maize fields where traps were hung. However, identification of pheromone from ECB females collected in maize stalks has demonstrated that E phenotype individuals can develop on maize in the United States, Switzerland, Hungary, and Italy (Roelofs et al., 1985; Peña et al., 1988). In addition, we confirmed that an ECB population collected on maize in Piacenza (Italy) belonged to the E strain (Frérot, unpublished data). Therefore, the E strain predominance in maize in these countries triggers further questions. First, are there any local populations in these places feeding on mugwort or hop? Second, what type of sex pheromone (E vs. Z) would these mugwort or hop populations use? Genetic studies would be of major interest to determine whether E maize populations from those places genetically differ from E hop and E mugwort races from France.

It is noteworthy that GC-MS results showed that mugwort and hop ECB individuals shared the same-sex pheromone blend composed of E11-14:OAc, Z11-16:OAc, and 14:OAc in similar ratios. Investigating maize populations in northern Italy, southern Switzerland, and Hungary, Peña et al. (1988) reported that most maize females belonging to the E type produced Z11-16:OAc in a proportion of 5–10% of E11-14:OAc. Our results also point out a correlation between the presence of Z11-16:OAc and the E phenotype. Thus, in Europe, the E phenotype of *O. nubilalis* is characterized by E11-14:OAc as a main component and Z11-16:OAc as a consistent minor component. The behavioral role of this latter component is not yet known. Field trapping with Z11-14:OAc/E11-14:OAc/Z11-16:OAc blends in the following ratios 3/97/1, 3/97/5, 3/97/20 did not reveal any effect of Z11-16:OAc

on male attraction (Peña et al., 1988). This common sex pheromone composition might enable cross-attraction and interbreeding between hop race and mugwort race.

These results support the description of genetic proximity between mugwort and hop races reported by Bourguet et al. (2000) and confirm that they resemble "a single genetic panmictic unit." In contrast, the genetic differentiation of ECB maize race from the mugwort and hop races can be related to the use of different pheromone blends. Both the description of males' pheromone response specificity (Glover et al., 1987) and wind tunnel results presented here show that homogamic mating is strongly promoted in each pheromone strain. The lack of hybrid females observed in comparison to Hardy–Weinberg equilibrium expectations further confirms the ECB pheromone system specificity. Such specificity is probably the major factor that maintains and may have initiated the genetic differentiation of the Z maize race and the E mugwort and hop races. From an evolutionary point of view, the results of this study are interesting with respect to *Ostrinia* spp. phylogeny. The maize, mugwort, and hop populations investigated were not polymorphic with respect to the Z or E pheromone status of individuals, and few hybrids were found on each host plant. The abundance of E individuals on mugwort and hop reinforces the hypothesis that the E strain is the primitive strain in *O. nubilalis.* Both points support the status of hop as ancestral host plant for *O. nubilalis*. Ishikawa et al. (1999) reported that (i) both *O. nubilalis* and *O. scapulalis* feed on *H. lupulus* and (ii) considered on the basis of molecular studies that these species have recently diverged. These points suggest that *O. nubilalis* and *O. scapulalis* may have originated from a common ancestor feeding on hop.

Second, some characteristics of the *O. nubilalis* mate location system are compatible with the scenario of a Z strain founded by a mutant Z female appearing in an E population. Because of the high level of intermale competition for access to mates, any mutant male would be out-competed by nonmutant competitors and would not mate. Thus, scenarios advocating the appearance of mutant males are unlikely to have occurred. Considering scenarios based on the appearance of a mutant female, one may expect a greater fitness from a mutant Z female appearing in an E population compared to a mutant E female appearing in a Z population. Because of the broader response window of E males (Glover et al., 1991), "a heterozygote female producing 35% Z isomer in an otherwise pure E strain population would be approximately a quarter as attractive to E strain males as homozygote E strain females, whereas a heterozygote female would hardly attract any males at all in a pure Z-strain population" (Löfstedt, 1990). This allows the emergence of a Z strain from a primitive E strain if the mutation is considered to spread in the populations from females, not from males. Further investigations are needed to compare the transmission probability of the mutant allele when it is hosted by a hybrid male and a hybrid female.

Taking into account the work of Hansson et al. (1987), Linn and Roelofs (1995) reported in their review that ECB males possess specific receptors for Z9-14:OAc, and females produce significant amounts of Z11-16:Acyl, the biosynthetic precursor. These two points led Linn and Roelofs (1995) to hypothesize that ECB (no detail is given on the pheromone strain considered) have lost the ability to produce Z9-14:OAc but still produce Z11-16:Acyl as a "vestigial" component. Because Z11-16:Acyl can also be the precursor for the Z11-16:OAc biosynthesis, and because in our samples E females produced a stable ratio of E11-14:OAc/Z11-16:OAc, we put forward the alternative hypothesis that the Z maize race has lost the ability to produce Z11-16:OAc. This component is still present in the E hop/mugwort races and would be considered a primitive character. This point also supports the hypothesis of the E strain precedence in ECB pheromone system evolution, but the opposite scenario cannot be rejected. Zhao et al. (1995) and Zhu et al. (1996) have shown functional similarities between the fatty acid reductase enzyme involved in pheromone production in the Z pheromone strain of *O. nubilalis* and the related species *O. furnacalis*, which use a Z12-14:Ac/E12-14:Ac blend. These similarities are in favor of the "primitive Z strain scenario."

Interestingly, pheromone polymorphism in the *Ostrinia* genus is described for *O. nubilalis*, *O. scapulalis* (Huang et al., 1997, 2002), and *O. furnacalis* (Guenée) (Huang et al., 1998), which are all polyphagous species (Ishikawa et al., 1999). Further work must establish whether genetic differentiation of host races and asymmetry of pheromone strain distribution among host plants is limited to *O. nubilalis*, or also to *O. scapulalis* and *O. furnacalis*. Host-related segregation of pheromone strains is not the general rule in Lepidoptera, and numerous moth species exhibit a high level of geographic variation (Linn and Roelofs, 1995). However, both in Lepidoptera and other insects, specific mate recognition systems (SMRSs) have been shown to vary among host plant or habitat races. In the larch budmoth (*Zeiraphera diniana*, Guenée; Lepidoptera, Tortricidae), two host races use different pheromone blends and are genetically different (Guerin et al., 1984; Emelianov et al., 1995). SMRS shifts related to habitat or host specialization are not surprising because specialization requires the onset of specific genetic combination, and alternative SMRS promote the conservation of such new genetic sets. However, whether SMRS shift precedes, follows, or reinforces host specialization is a hard question to address. The theoretical framework for both possibilities exists within the traditional speciation process proposed by Mayr (1994) on one hand, and the innovative view of Paterson (1985) on the other hand.

Host specialization is often evoked as a driving force for species splitting into host races and sympatric ecological speciation (Diehl and Bush, 1984; Tauber and Tauber, 1989; Schluter, 2001; Via, 2001), but such specialization has to be expressed through host-associated adaptations in response to divergent selection. For example, in *Rhagoletis pomonella* (Walsh, Diptera, Tephritidae), the hawthorn race and the apple race have distinct differences in developmental timing that

allow adult flies to emerge when their respective hosts are available (Feder et al., 1993). Hawthorn-origin flies also exhibit a preferential wind-tunnel response for hawthorn fruit volatiles compared to apple volatiles (Nojima et al., 2003). When insect host races exist, individuals can experience reduced fitness when reared on each other's host (Tauber and Tauber, 1989). In *O. nubilalis*, polyphagy has been known for a long time, but host-associated adaptation in *O. nubilalis* pheromone strains is not documented. The few data available concern comparative studies on bivoltine E, and uni- and bivoltine Z larvae artificially established on unusual host plants such as snap beans and apple trees (Straub et al., 1986; Eckenrode and Webb, 1989). These experiments demonstrate that the bivoltine E strain (originating from maize) presents a greater potential for infestation of new host plants. However, no conclusions can be drawn with regard to a reversed lower adaptation to maize.

In summary, divergence in sex pheromone composition and host-plant use were shown to be intimately linked in ECB populations in France. This finding is consistent with the genetic structure of ECB populations described in France and highlights that ECB is a pertinent model for studying gene flow and speciation processes. Because the key to speciation may lie "in an evolutionary synergism between ecological specialization and reproductive isolation," (Via et al., 2000), it is now of interest to investigate the specific nature of this link between pheromone strains—which enhance assortative mating—and host plants that may represent divergent ecological selection pressure.

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ATTRACTION OF THE LARVAL PREDATOR *Elater ferrugineus* TO THE SEX PHEROMONE OF ITS PREY, *Osmoderma eremita*, AND ITS IMPLICATION FOR CONSERVATION BIOLOGY

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Abstract—*Elater ferrugineus* is a threatened click beetle inhabiting old hollow trees. Its larvae consume larvae of other saproxylic insects including the threatened scarab beetle *Osmoderma eremita*. Recently, (*R*)-(+)-γ-decalactone was identified as a male-produced sex pheromone of *O. eremita*. Here we present evidence that *E. ferrugineus* adults use this odor as a kairomone for location of their prey. In field trapping experiments, significantly more trapping events of *E. ferrugineus* beetles were observed in Lindgren funnel traps baited with (*R*)-(+)-γ-decalactone than in control traps (20 vs. 1, respectively). Analyses of headspace collections from *E. ferrugineus* beetles indicate that the predator itself does not produce the substance. Both sexes were attracted to the prey pheromone, suggesting that *E. ferrugineus* males use the odor as an indirect cue for location of mates or of the tree hollows, which make up their habitat. When compared to pitfall traps, the Lindgren system was significantly more effective in trapping *E. ferrugineus*, and no difference could be established for *O. eremita*, showing the high potential to use odor-based systems to catch both species. We suggest that (*R*)-(+)-γ-decalactone could be used as a master signal in monitoring programs for these vulnerable beetle species, which are both regarded as indicators of the associated insect fauna of the threatened habitat of old hollow trees.

Key Words—*Elater ferrugineus*, *Osmoderma eremita*, Coleoptera, (*R*)-(+)-γ-decalactone, kairomone, chemical communication, monitoring, conservation.

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INTRODUCTION

Predatory insects frequently rely on olfactory cues for detection and location of their prey, including exploitation of compounds that are used by their prey for intraspecific communication (Aldrich, 1999). Several insect species that prey on bark beetles are attracted to traps baited with aggregation pheromone components produced by their prey (Bakke and Kvamme, 1981; Wood, 1982; Raffa and Dahlsten, 1995). Until today, research on these kinds of chemically mediated interactions has focused on systems where the prey is regarded as a pest species in order to find odor cues attractive to natural enemies for use in control programs. Few studies, if any, have investigated these interactions with focus on rare species pairs and the potential to use chemical signals in species conservation programs.

The scarab beetle *Osmoderma eremita* Scopoli (Coleoptera: Scarabaeidae) is strongly linked to old, hollow deciduous trees (Luce, 1996; Ranius and Nilsson, 1997). In Sweden, oak (*Quercus robur*) is the main habitat for *O. eremita* (Palm, 1959), but in other parts of Europe the species can be found in a large number of hollow-forming deciduous trees (Luce, 1996). As the habitat of old, hollow trees has decreased during the last centuries, the associated insect fauna, including *O. eremita*, is today threatened all over Europe (Hannah et al., 1995; Nilsson, 1997). The population ecology and dispersal pattern of *O. eremita* has received much attention (Ranius, 2001; Ranius and Hedin, 2001; Hedin and Ranius, 2002) because of its status as an indicator species (Ranius, 2002a) and its high conservation priority according to the European Union's Habitat Directive (Anonymous, 1992).

Recently, the male-produced sex pheromone of *O. eremita* was identified as (*R*)-(+)-γ-decalactone (Larsson et al., 2003). This buttery or fruity odor is released in large amounts by males and can be detected by the human nose at a distance of several meters. During field trapping of the species, a few individuals of the threatened click beetle *Elater ferrugineus* L. (Coleoptera: Elateridae) were also found in odor-baited traps. As the larva of this polyphagous species attacks larvae of other saproxylic insects including *O. eremita* (Hansen, 1966; Dajoz, 2000), our observations suggested that *E. ferrugineus* might use the sex pheromone emitted by *O. eremita* males as a kairomone to facilitate prey location. If shown to be true, (*R*)-(+)-γ-decalactone could be a key factor in the habitat and prey selection of *E. ferrugineus*, and would also be a potential master signal in monitoring programs for both species.

In this study, we investigated (i) if *E. ferrugineus* adults use (*R*)-(+)-γ-decalactone for location of *O. eremita* prey, (ii) if this odor is used as a general cue by other saproxylic beetles in habitat selection, and (iii) the effectiveness of an odor-based system in trapping *E. ferrugineus* and *O. eremita* compared to pitfall traps to evaluate the potential to use (*R*)-(+)-γ-decalactone for monitoring purposes.

METHODS AND MATERIALS

Chemicals and Dispensers. Synthetic (*R*)-(+)-γ-decalactone, the sex pheromone of *O. eremita*, was purchased from Sigma-Aldrich, Germany. As dispensers, cotton dental rolls (Celluron, Paul Hartmann, S.A., France) impregnated with 400 μl of neat (*R*)-(+)-γ-decalactone and inserted into 2-ml glass vials were used. Previous laboratory experiments revealed a stable release rate of approximately 2 mg per day of the compound during a 45-day period (unpublished data), which was longer than an average field season, i.e., the time between the first and last catch of adults in this area according to Ranius (2001).

Field Trapping. Field experiments were conducted in Bjärka-Säby (58° 16′ N, 15°46′ E) and Brokind (58°12′ N, 15°40′ E), south of Linköping, Sweden, from July 10–August 12, 2002. The study area included five large stands of old hollow oaks, housing one of the largest populations of *O. eremita* and *E. ferrugineus* in Sweden (Nilsson and Baranowski, 1994; Ranius, 2001, 2002b; Ranius and Hedin, 2001). All traps collected beetles without killing them and, apart from some *E. ferrugineus* individuals used for further analysis (see below), they were released again after examination. Thus, some individuals may likely have been trapped more than once (cf. Ranius, 2001). The given number of beetles trapped, therefore, reflects the number of trapping events rather than the total number of individual beetles.

Lindgren Traps. Lindgren funnel traps (Phero Tech Inc., Delta, British Columbia, Canada) were used for trapping of beetles using (*R*)-(+)-γ-decalactone as a lure. For each replicate ($N = 20$), one trap baited with (*R*)-(+)-γ-decalactone and one control trap without the odor were suspended at least 4 m apart in or close to trees known from earlier field studies to contain *O. eremita* (Ranius, 2001). The number of traps used is shown in Table 1. Traps were checked every second day for presence of *O. eremita*, *E. ferrugineus*, and other beetles associated with hollow oaks. The positions of traps within replicates were changed every fourth day. Trapping events of beetles in Lindgren traps were compared using a binomial test (Siegel and Castellan, 1988), against the null hypothesis that individuals would be equally attracted to traps with and without the odor.

Pitfall Traps. In addition to odor-baited traps, pitfall traps were used to study the co-occurrence of *E. ferrugineus* and *O. eremita* in tree hollows and to measure how effective the odor-based system was in trapping these beetles compared to pitfall traps. Empty glass or plastic jars (diam > 7 cm, height > 6 cm) were placed into the wood mold (fragmented, often powdery rotten wood mixed with fungi, fragments of dead insects, remains of bird nests, etc.) in cavities of 45 trees in the same areas. Each tree had only one main cavity in which one trap was placed, except for one tree with two independent hollows with one trap in each, giving a total of 46 traps (Table 1). Jars were placed with their opening at the level of the wood mold surface and checked every second day. The trapping efficacy of

TABLE 1. TRAPPING EVENTS OF *Elater ferrugineus* AND *Osmoderma eremita* IN DIFFERENT STUDY AREAS DURING 2002 USING LINDGREN TRAP ($N = 20$) AND PITFALL TRAPS ($N = 46$)

		E. ferrugineus			*O. eremita*		
		Lindgren trap			Lindgren trap		
Area	N[a]	Odor	Control	Pitfall trap	Odor	Control	Pitfall trap
Hjorthägnet	3, 8	5	0	0	0	0	10
Bjärka by	6, 12	5	0	3	21	1	129
Bjärka äng	2, 10	1	0	2	5	0	6
Storängen	2, 9	3	0	1	7	2	52
Brokind	7, 7	6	1	4	27	2	80
Total		20	1	10	60	5	277
Mean ± SD[b]		1.1 ± 0.5		0.2 ± 0.2	2.7 ± 1.6		6.0 ± 5.1

[a] Number of replicates used for Lindgren traps and pitfall traps, respectively.
[b] Means of the average number of trapping events per replicate from each area. The number of trapping events per replicate is significantly higher for *E. ferrugineus* in odor-baited Lindgren traps compared to pitfall traps, while no significant difference could be detected for *O. eremita* (Wilcoxon's signed ranks test: $P < 0.05$).

the two systems was compared using Wilcoxon's signed ranks test based on the average number of trapping events per replicate from each area.

Although there are slight differences in body size between the sexes (Hansen, 1966), males and females of *E. ferrugineus* can only be distinguished reliably by dissecting their reproductive organs. However, as the species is vulnerable and occurring in low numbers, sex determination of beetles found in odor-baited traps was restricted to include six individuals caught in 2002 and two additional individuals caught during previous field experiments to minimize the negative effects of removing individuals from the population.

Headspace Sample Analysis. To exclude the possibility that the attraction of *E. ferrugineus* to (*R*)-(+)-γ-decalactone in the field was due to this substance also functioning as a pheromone for this species, the presence of the odor in headspace collections of beetles was analyzed. Live trapped beetles were frozen upon collection and brought to the laboratory in Lund within 2 weeks after capture. This analysis included the six individuals later used for sex determination of beetles attracted to Lindgren traps in 2002 (see above), tested in groups of two, and four additional individuals found in pitfall traps tested together. Volatile compounds emitted from dead beetles were collected for 7 hr in a closed-loop system using a pump (Parker Hannifin Corp. Sharon, MA, USA) with a capacity of 0.7 l/min. Compounds were trapped on 1.5 mg charcoal filters (Brechbühler AG, Switzerland), eluted in 30 μl of acetone, and concentrated under N_2 before analysis.

Analyses of samples were performed using an Hewlett–Packard 5890 series II gas chromatograph equipped with an HP-Innowax polar capillary column (30 m $\times$ 0.25 mm i.d. with a 0.25-μm film thickness), linked to an Hewlett–Packard 5972 Mass Selective Detector (MSD). Helium was used as carrier gas at a constant flow of 40 cm/sec. The temperature of the injector was 225°C. Column temperature was maintained at 40°C for 2 min after injection and then linearly increased to 230°C at a rate of 5°C/min. The column used did not allow enantiomeric separation of compounds and, therefore, only presence of γ-decalactone in samples could be determined. Presence of the compound in headspace samples of beetles was checked by MS analysis and by retention time match with synthetic (*R*)-(+)-γ-decalactone purchased from Sigma-Aldrich, Germany. A peak in the total ion chromatogram, corresponding to that of synthetic (*R*)-(+)-γ-decalactone at a detection limit of 1 ng, was used to determine presence of the compound in a sample. The occurrence of a prominent mass peak typical for lactones at m/z 85 was used to distinguish the compound from others with similar retention times.

RESULTS

Lindgren Traps

E. ferrugineus. Significantly more trapping events of *E. ferrugineus* were observed in traps baited with (*R*)-(+)-γ-decalactone compared to control traps (20 vs. 1, respectively, $P < 0.001$, Figure 1). On the basis of genital examination of six individuals found in Lindgren traps with lures during 2002, three of these were identified as males and three as females. The two individuals trapped during the previous field season included one male and one female. Thus, both sexes of the predator seem to be attracted to the male-produced sex pheromone of *O. eremita*. In the majority of cases only single individuals of the predator were found in odor-baited traps, However, in two cases, two individuals, and in two cases, three individuals, respectively, were found together. In addition, *E. ferrugineus* beetles were found together with a female *O. eremita* in an odor-baited trap on two occasions.

O. eremita. More trapping events of *O. eremita* females were observed in odor-baited traps than in control traps (56 vs. 5, respectively, $P < 0.001$, Figure 1). In contrast, only four trapping events of males were observed in pheromone-baited traps (and none in control traps), which is consistent with earlier trap catch data showing weak attraction of males to the odor (Larsson et al., 2003).

Other Beetles. Saproxylic beetle species such as *Allecula morio* Fabricius (Alleculidae), *Prionychus ater* Fabricius (Alleculidae), and *Pseudocistela cerambboides* L. (Alleculidae) were also found in Lindgren traps, but in low numbers (maximum three individuals in either treatment or control) and no significant attraction of these species to the odor could be established.

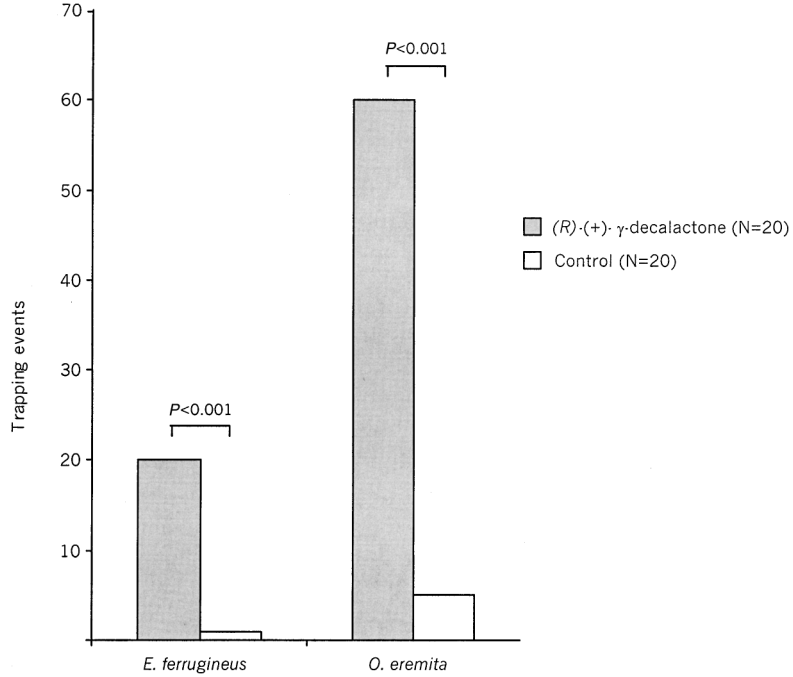

FIG. 1. Trapping events of *E. ferrugineus* and *O. eremita* beetles (both sexes) in Lindgren traps with or without (*R*)-(+)-γ-decalactone. Both species were significantly more attracted to odor-baited traps compared to control traps according to a binomial test by Siegel and Castellan (1988).

Pitfall Traps

E. ferrugineus. A total of 10 trapping events of the predator were noted in pitfall traps during the field season (Table 1). In six trees, only a single *E. ferrugineus* beetle was trapped; in one tree two independent trapping events occurred, and in another tree, two beetles were found together in the same trap. In five of the eight trees where *E. ferrugineus* was found, *O. eremita* beetles were also found during the same field season. The number of trapping events of *E. ferrugineus* in pitfall traps was, however, too small for meaningful statistical correlation between the species based on this material. The odor-based system was more effective in trapping beetles compared to pitfall traps (Lindgren traps: 1.1 ± 0.5 trapping events per trap, pitfall traps: 0.2 ± 0.2 trapping events per trap, $P < 0.05$).

O. eremita. In total, 278 trapping events of *O. eremita* were observed (201 males and 77 females). In contrast to *E. ferrugineus,* no difference in trapping efficacy of this beetle was observed between Lindgren and pitfall traps (Lindgren

traps: 2.7 ± 1.6 trapping events per trap, pitfall traps: 6.0 ± 5.1 trapping events per trap, $P > 0.10$).

Headspace Analysis. No detectable amounts of the lactone could be found in headspace extracts from any of the six *E. ferrugineus* beetles found in Lindgren traps and used for sex determination or from the four additional beetles (two of each sex) found in pitfall traps when checked by coupled GC–MS (detection limit: 1 ng).

DISCUSSION

Our results show that adult males and females of the larval predator *E. ferrugineus* are attracted to (*R*)-(+)-γ-decalactone, the sex pheromone produced by its prey, *O. eremita* (Figure 1). The observation suggests that this predator uses the odor as a kairomone to facilitate prey location. The substance was not detected in headspace collections from any sex of the predator, although the sample size was small due to restricted sampling of beetles. Apart from the absence of this odor in samples from beetles, two additional circumstances argue against the function of (*R*)-(+)-γ-decalactone as an intraspecific signal in *E. ferrugineus*. First, in all click beetle species investigated so far, females produce the sex pheromone to attract males, and females are not attracted to the odor themselves (Borg-Karlsson et al., 1988; Yatsynin et al., 1996; Tóth et al., 2002). Second, all click beetles studied to date use terpenic esters as pheromone components, which differ greatly in molecular structure from lactones.

Adult *E. ferrugineus* of both sexes seem to exploit the sex pheromone released by adult males of the prey as an indirect cue to find trees that have a high probability of housing *O. eremita* larvae in the next generation(s). This is different from, e.g., clerid beetles of the genus *Thanasimus*, that prey on both bark beetle adults and their brood (Frazier et al., 1981; Weslien, 1994). Although the number of trapped individuals used for sex determination was small, the attraction of male *E. ferrugineus* to odor-baited traps seems to be similar to that of females, suggesting that males use the prey pheromone as an indirect cue to find females of their own species for mating. According to Hansen (1966), *E. ferrugineus* swarms during evenings and nights. Its mating behavior is poorly studied, however, presumably because the species is rare.

In five out of seven trees where *E. ferrugineus* beetles were found in pitfall traps, *O. eremita* beetles were also observed during the field season. Although the total number of beetles trapped was too low for any meaningful statistical analysis, it suggests a strong association of the predator to its prey. On the basis of the dataset presented by Ranius (2002b), a strong correlation of occupancy was observed between the two species, with fragments of *E. ferrugineus* found almost exclusively in trees with fragments of *O. eremita* (Ranius, personal communication). Trap catch

data also indicate an association on a temporal scale. During the previous field season with relatively low trap catches of *O. eremita* adults (Larsson et al., 2003), only two *E. ferrugineus* beetles were found in odor-baited traps, and no beetles were found in pitfall traps. In contrast, during this study much higher trap catches of the predator as well as the prey were observed for both trapping systems. The impact of *E. ferrugineus* on brood mortality in *O. eremita* is unknown, and long-term studies are needed to analyze the degree of correlation among populations of these species.

As *O. eremita* is regarded as an indicator of species richness associated with tree hollows (Anonymous, 1992; Ranius, 2002a), one could hypothesize that its sex pheromone could be exploited also by saproxylic beetle species that do not prey upon *O. eremita*, thereby functioning as a general cue in habitat selection. Air sampling experiments in tree hollows in the experimental area have shown that the amounts of the pheromone produced by male *O. eremita* are high enough to be detected even by traditional GC–MS analysis (Svensson et al., in press), potentially making this odor an ideal chemical signal for locating suitable habitats. Several beetles, which have been shown to be associated with *O. eremita* in tree hollows (Ranius, 2002a), were found in odor-baited traps. However, they occurred in low numbers and significant attraction to the odor could only be established for *E. ferrugineus*. Identification of chemical cues used by other saproxylic beetles for detection and location of suitable trees is needed to facilitate monitoring of these species.

The odor-based system was significantly more effective in trapping *E. ferrugineus* when compared with pitfall traps, whereas no significant difference in trapping efficacy between systems was observed for *O. eremita* (Table 1). However, the mean number of trapping events in *O. eremita* was higher for pitfall traps than for odor-baited Lindgren traps, and the lack of a significant difference may have been due to the low number of replicates used in the analysis. In spite of this, the results indicate that $(R)-(+)$-γ-decalactone is a potent attractant for both *O. eremita* females (Larsson et al., 2003) and *E. ferrugineus*. Trapping events using Lindgren traps require that the beetle has dispersed from its original habitat. The proportion of beetles accessible with odor-baited traps outside tree hollows, thus, represents only a fraction of the total population, in contrast to trapping events inside tree hollows using pitfall traps. On the other hand, trapping events in pitfall traps have higher information value, as they provide an unambiguous association with specific trees and can be used to estimate population sizes (Ranius, 2001). No additional chemical cues derived from tree hollows seem to be necessary for the attraction of beetles to the odor-baited traps. Thus, $(R)-(+)$-γ-decalactone could be used as a master signal in monitoring programs for both beetle species with reasonable efficiency. The possibility to increase catches inside tree hollows by adding $(R)-(+)$-γ-decalactone to pitfall traps has not been investigated for either species.

The practical goal of research on semiochemicals of forest insects has been the potential to use these compounds in control programs to reduce populations of economically important pests such as bark beetles (Borden, 1997). Considering the increased public and academic interest in species conservation and biodiversity during the last decades, surprisingly little effort has been made to study chemical communication of threatened insects and identify key substances which could contribute to the improvement of conservation strategies for these species. Difficulties are involved in studying rare species due to low numbers of individuals present in an area, which may constrain the analysis, and long-term studies are probably needed in many cases. However, this study shows that the use of behavior-modifying odors can give the conservation biologist a useful tool when investigating threatened insect species.

The habitat of old hollow trees, used by numerous saproxylic insects, has decreased severely because of fragmentation and destruction (Hannah et al., 1995; Nilsson, 1997; Eliasson and Nilsson, 2002). Today, both *E. ferrugineus* and *O. eremita* that are dependent on hollow trees occur at few sites in Sweden (Nilsson and Baranowski, 1994) and are considered vulnerable (Gärdenfors, 2000). Both species are also suggested as indicators of the species richness of the habitat of hollow trees (Nilsson et al., 2001). Thus, by establishing reliable monitoring systems for these beetles, the overall species diversity may be estimated, which is of importance when selecting areas for conservation and evaluating management measures.

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CUTICULAR HYDROCARBONS AND AGGRESSION IN THE TERMITE *Macrotermes Subhyalinus*

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Abstract—Cuticular hydrocarbons are among the prime candidates for nestmate recognition in social insects. We analyzed the variation of cuticular hydrocarbons in the termite species *M. subhyalinus* in West Africa (Comoë National Park) on a small spatial scale (<1 km). We found considerable variation in the composition of cuticular hydrocarbons among colonies, with four distinct chemical phenotypes. Different phenotypes occurred within each of the four habitats. The difference between these phenotypes is primarily due to unsaturated compounds. A clear correlation between the difference of the hydrocarbon composition and the aggression between colonies was found. This correlation also holds in a multivariate analysis of genetic similarity (measured by AFLPs), morphometric distances (measured by Mahalanobis-distances), as well as geographic distances between colonies. In a more detailed analysis of the correlation between the composition of cuticular hydrocarbons and aggression, we found that no single compound is sufficient to explain variation in aggression between pairings of colonies. Thus, termites seem to use a bouquet of compounds. Multiple regression analysis suggested that many of these compounds are unsaturated hydrocarbons and, thus, may play a key role in colony recognition.

Key Words—Nestmate recognition, cuticular lipids, AFLP, DNA-fingerprinting, relatedness, morphometrics, speciation, chemical ecology.

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INTRODUCTION

All social insects have the need to distinguish nestmates from nonnestmates (reviewed in Hölldobler and Michener, 1980; Breed and Bennett, 1987; Hölldobler and Wilson, 1990; Breed, 1998). For nestmate recognition, individuals search for a label when comparing one another and compare this label with a template. If label and template match, the two individuals will recognize each other as nestmates. This implies the acquisition, expression, or transfer of a label, and the learning of a template. Different models of nestmate recognition have been proposed (Carlin and Hölldobler, 1986; Hölldobler and Wilson, 1990; Smith and Breed, 1995). (1) The individualistic model assumes that labels are genetically determined and nontransferable. (2) The labels are acquired from the environment. (3) The Gestalt model assumes that the labels are a mixture of substances transferred among nestmates. (4) Finally, labels may be distributed from the queen to all nestmates.

Intraspecific nestmate recognition and associated mechanisms have been thoroughly studied in Hymenoptera. Intraspecific recognition cues, including cuticular hydrocarbons, are thought to be transferred among nestmates via intermediary nest structures such as comb wax in the honey bee *Apis mellifera* (Smith and Breed, 1995; Breed et al., 1998) or via the paper nest in *Polistes* (Gamboa et al., 1986; Dani et al., 1996; Gamboa et al., 1996). Thus, *Apis* and *Polistes* seem to follow the Gestalt model. Although cues for colony recognition have not yet been clearly identified in the sweat bee *Lasioglossum zephyrum*, they are not transferred among nestmates, which corresponds to the individualistic model (Greenberg, 1979; Buckle and Greenberg, 1981; Smith and Breed, 1995). In various ant species, recognition mechanisms may involve cues collected from the environment (e.g., Obin, 1986). However, evidence supporting the individualistic model (e.g., Mintzer and Vinson, 1985), the Gestalt model (e.g., Soroker et al., 1994), or recognition being mediated by queen derived cues (e.g., Carlin and Hölldobler, 1986) has also been found in ants.

It is generally believed that chemical cues are the labels involved in nestmate recognition. Cuticular hydrocarbons are assumed to be of major importance (reviewed e.g., in Smith and Breed, 1995). In ants, the search for recognition cues has almost exclusively concentrated on cuticular hydrocarbons (Bonavita-Cougourdan et al., 1987; Henderson et al., 1990; Nowbahari et al., 1990; Soroker et al., 1994). In termites, research on nestmate recognition has focused on behavioral aspects (reviewed by Thorne and Haverty, 1991; Shelton and Grace, 1996; Clément and Bagnères, 1998), but chemical cues have rarely been investigated. Nevertheless, it was repeatedly shown that cuticular hydrocarbon composition can vary considerably between termite colonies in close proximity (e.g., Haverty et al., 1988, 1996b,c; Bagine et al., 1994; Brown et al., 1996; Kaib et al., 2002). Some studies have combined the analysis of cuticular hydrocarbons with behavioral tests. These studies provide some evidence that cuticular hydrocarbons are correlated with the

recognition process (Howard et al., 1982; Haverty and Thorne, 1989; Bagnères et al., 1991; Takahashi and Gassa, 1995; Kaib et al., 2002). However, most of these studies concentrated on species recognition (but see Kaib et al., 2002). Finally, three studies suggested that the cues for nestmate recognition have a genetic basis, although the chemical nature of the labels themselves were not analyzed (Adams, 1991; Husseneder et al., 1997, 1998).

In the present study, we combine results derived from the chemical analysis of cuticular hydrocarbons, aggression tests between major workers from different colonies, genetic relatedness between colonies (AFLP-fingerprinting), and morphometric differences between minor soldiers from colonies in the higher termite *Macrotermes subhyalinus* (Isoptera, Termitidae). We include morphometric differences in our study, as individuals may recognize nestmates from their morphological similarity, which is generated by the similar genetics of nestmates, but also by the common environment they share. With these data sets we want to answer the following questions:

1. Is there a sufficient difference in the composition of cuticular hydrocarbon mixtures among colonies to allow for nestmate recognition? 2. Is the difference in the composition of cuticular hydrocarbons correlated with the behavioral interaction between colonies? 3. Are genetic or environmental factors more important than the cuticular hydrocarbon composition for predicting the level of aggression between colonies?

METHODS AND MATERIALS

Species and Study Site. According to Ruelle (1970), *M. subhyalinus* occurs across the northern savannah region from West to East Africa. In West Africa, its distribution stretches from coconut plantations along the coast to the semiarid northern regions. The species builds large earthen mounds (up to 1 m high) that, in contrast to East African *M. subhyalinus*, do not have open ventilation chimneys. It may be that the western and eastern populations are different species, a conclusion that is supported by mt-DNA sequences (M. Kaib, unpublished results). Hence, in this study the species name is provisional and may change when a revision of the genus *Macrotermes* becomes available. Colonies of the investigated species are monocalic and, in most cases, monogynous. *M. subhyalinus* feeds on rotten wood, dead leaves, and dry grass.

The study was carried out in the southeastern part of Comoë National Park ($8^\circ 45' N - 3^\circ 47'$ W; Ivory Coast, West Africa), an area characterized by a wet Guinea Savannah with 1100–1700 mm precipitation per year (Korb, 1997). The sampling area was situated in loose island forests. We selected 10 colonies within 4 different focus locations (Figure 1). Distances between adjacent locations ranged from 190 m–790 m. As an outgroup, one colony (R1; not shown in Figure 1) was

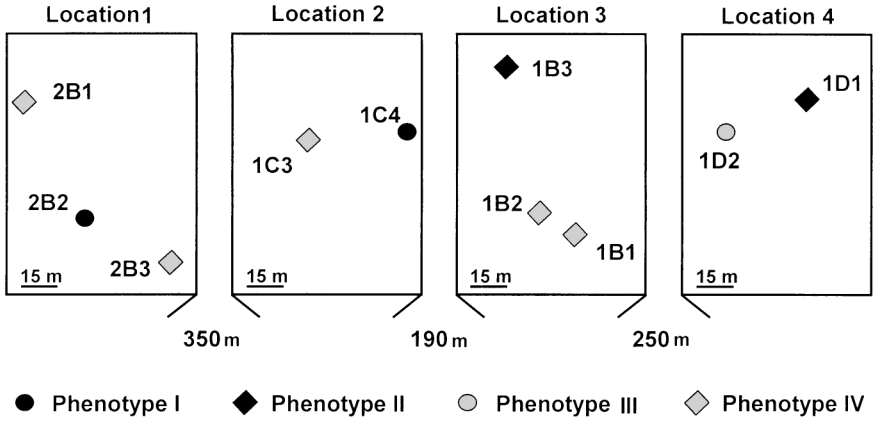

FIG. 1. Map of the 10 colonies within the 4 sampled locations at the focus area. Note that phenotypes are not clustered according to location.

collected about 20 km away from the main sampling area. This colony was included in the chemical analysis (Figure 2), but not in the evaluation of the relationship between aggression and hydrocarbons.

Chemical Analysis of Cuticular Hydrocarbons. In March 1997, 12 major workers were collected from each of the 11 colonies (except for 1D1: 6 major workers; for colony labels and distribution see Figure 1). Individuals were washed immediately after collection for 15 min in 100 μl *n*-hexane (Merck 1.04391). Cuticular rinses were evaporated to dryness in order to eliminate volatile constituents. For chemical analysis, the rinses were reconstituted with *n*-hexane, and aliquots were assayed by gas chromatography (Hewlett Packard 5890, equipped with a flame ionization detector). Separation of components was performed on a fused silica capillary column (DB-1; 30 m by 0.32 mm i.d.; 0.11 μm film thickness). The carrier gas was helium at a flow rate of 1.5 ml/min. The injection mode was splitless for the first 60 sec after injection. For separation of hydrocarbons, the oven temperature was programmed from 160 to 260°C at 2°C/min, thereafter to 300°C at 5°C/min, and then held isothermally for 15 min. FID signals were acquired and quantified by HP ChemStation. Peaks from different chromatograms were classified by comparison of linear retention indices calculated on the basis of an *n*-alkane series from eicosane to hexatriacontane.

We used 40 gas chromatographic peaks that were derived from hydrocarbons. The percentages of each peak were calculated on the basis of the total counts of all 40 peaks. The matrix resulting from the percentages of the peaks from all analyzed individuals was subjected to further statistical analyses. We are aware of the fact that in some cases, peaks assigned to a retention index may include more than one hydrocarbon. Therefore, our distance measures are conservative estimates.

To quantify differences of chemical compositions between sampled individual major workers, we calculated a matrix of Nei-distances as described by Kaib et al. (1991). Other methods of quantifying the differences between compositions (see for example Kaib et al., 2002) generated the same patterns and, thus, we report only the analyses of the Nei-distances. Chemical distances between colonies were estimated by the mean of all possible pairwise combinations of the distances between individual major workers of the different colonies.

We identified individual hydrocarbons by coupled GC/MS (Blomquist et al., 1987; Nelson et al., 1980; Page et al., 1990a, b). For selected samples, structural analysis was performed on a VG 70-250 SE mass spectrometer connected to a Hewlett Packard HP 5890 GC. Aliquots containing 1–2 μl of an extract were applied to the column under the conditions stated above, apart from the oven temperature program (80°C; 1.5 min hold; at 40°C/min to 160°C; then at 2.5°C/min to 280°C). The mass spectrometer was operated in electron impact ionization mode (70 eV, 500 μA ionization current, 200°C source temperature) and scanned from *m/z* 600 to *m/z* 35 at a rate of 0.7 sec/decade with an interscan time of 0.2 sec. For the determination of the double bond positions of unsaturated hydrocarbons, dimethyl disulfide (DMDS) derivatives were prepared following the protocol provided by Kaib et al. (2000).

Aggression Among Colonies. Jmhasly and Leuthold (1999) report behavioral data (aggression indices) derived from the same colonies that we sampled for the analyses of chemical, morphometric, and genetic data. These behavioral data, which were obtained in 1996, were also used in the present study. Quantitative behavioral tests were done by pairing two groups of five major workers each from different colonies. During a period of 5 min, aggressive interactions were scored in 30-sec intervals. Aggression comprised grasping, nipping, and biting. For each colony, 10 replicates were done. An average of the 10 scores per test and the 10 replicates was used to calculate the aggression index that had a maximum range from 0 to 10. See Jmhasly and Leuthold (1999) for further details. They were not able to carry out experiments for all 45 possible combinations of the 10 colonies, thus, only 20 combinations are available.

Morphometrics. A total of 15 linear parameters of minor soldiers (collected in March 1997 and stored in 70% ethanol) were measured in 10 individuals from each of the 10 investigated colonies: head capsule length, distance from the fontanelle to the anterior margin of the head capsule, distance from the fontanelle to the posterior margin of the head capsule, distance between left and right mandible bases, distance from the fontanelle to the left mandible basis, maximal head capsule width, maximal pronotum width, pronotum length, maximal mesonotum width, maximal metanotum width, maximal head capsule height, left mandible length, length of left hind femur, length of left hind tibia, and length of left hind tarsus. Prior to analysis, all parameters were log-transformed. The data sets were subjected to canonical discriminant analysis by defining all individuals measured

from a colony as a group. Morphometric distances between termites from different groups (colonies) were calculated as Mahalanobis-distances.

AFLP-Fingerprinting. From each of the 10 colonies, four major workers (collected in March 1997 and stored in 100% ethanol) were used for genetic analysis. DNA was extracted from the heads of individual termites using DNeasy Tissue Kit (QIAGEN), following manufacturer's instructions applying 50-μl H_2O at the final elution step. The use of heads only minimizes the risk of contamination from gut symbionts of the termites. Amplified fragment length polymorphisms (AFLP; Vos et al., 1995) were generated as follows: First, in a total of 11 μl, 5.5 μl of DNA solution were restricted with 5 U *Eco*RI and 1 U *Mse*I, and ligated to respective adapters by 67 U T4 DNA ligase in the presence of 1 $\times$ T4 DNA ligase buffer, 0.05 mg/ml BSA, and 50 mM NaCl at 37°C for 2 hr. Second, after 1:2 dilution, 5 μl of the restriction-ligation reaction were used for preselective amplification in a total volume of 20 μl with 0.8 U *taq* polymerase (MBI Fermentas), 1x PCR buffer with $(NH_4)_2SO_4$ provided by the manufacturer, 1.5 ng/μl of *Eco*RI and *Mse*I preselective primers, 200 μM dNTPs, and 1.5 mM $MgCl_2$. PCR was performed in a Primus 96 thermocycler (MWG Biotech) programmed for 72^{o}C, 2 min followed by 30 cycles of (94^{o}C, 20 sec; 56^{o}C, 30 sec; 72^{o}C, 2 min) and 60^{o}C, 30 min. Third, after 1:20 dilution, 6 μl of the diluted amplification reaction were used for selective amplification with the same reaction conditions using 0.25 ng/μl *Eco*RI selective primer labeled with a fluorescent dye and 1.5 ng/μl *Mse*I selective primer and a "touch down" PCR protocol of 94°C, 2 min, followed 10 cycles of (94°C, 20 sec; 66°C, 30 sec, decrease 1°C per cycle; 72°C, 2 min), 20 cycles of (94°C, 20 sec; 56°C, 30 sec; 72°C, 2 min), and 60°C, 30 min. Subsequently, fragments were separated on an ABI 310 genetic analyzer. We tested 64 different primer combinations in four individuals, from which four primer combinations were chosen for further analysis according to number and size distribution of polymorphic fragments: *Eco*RI-ACT/*Mse*-CTT, and *Eco*RI-ACT/*Mse*-CAA, *Eco*RI-ACG/*Mse*-CTT, *Eco*RI-ACC/*Mse*-CAA. In total, these primers yielded 403 dominant loci, 92 of which were monomorphic. Only fragments that amplified consistently across samples were scored in a presence–absence matrix. From this matrix, genetic similarities among individuals were calculated using the Jaccard-index (Rohlf, 1990; other indices for presence/absence data produce the same results). For comparisons of colonies, the mean of all possible pairwise combinations between individuals from all combinations of colonies were calculated.

Statistical Analyses. To summarize the patterns in the 114 $\times$ 114 matrix of chemical distances between individuals, we performed a principal coordinate analysis. Cuticular hydrocarbon compositions, the aggression tests, morphometrics, and fingerprinting, as well as geographical distance between colonies were compared by matrix correlations. One-tailed error probabilities were estimated via permutations (5000 permutations) by a program that takes missing values into account (see also Manly, 1997).

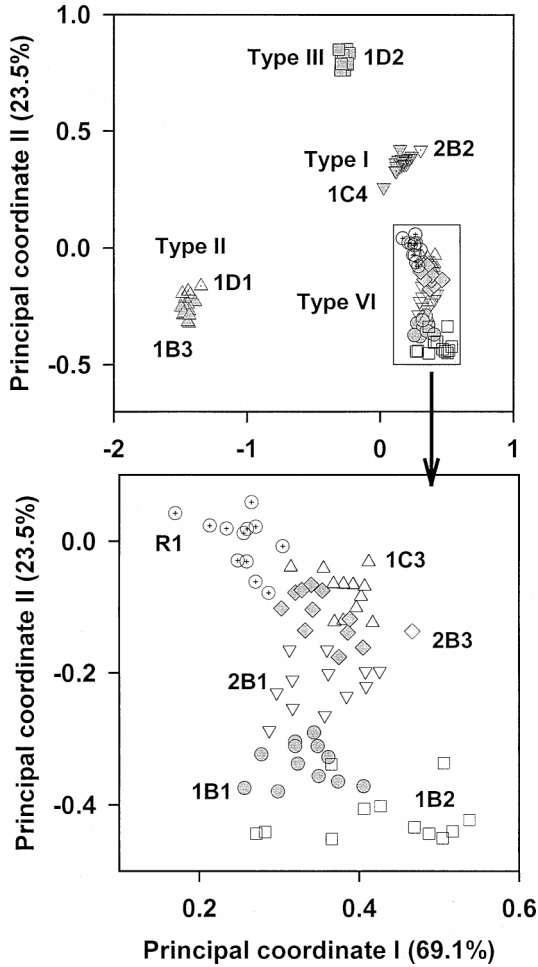

FIG. 2. Principal coordinate analysis of the differences among hydrocarbon compositions of 126 individuals from 11 colonies (including the outgroup colony R1, see Figure 1; differences measured by Nei-distances; Rohlf, 1990). Individuals from the same colony have the same symbol. Note the four distinct chemical phenotypes. The lower plot zooms the ordination of phenotype IV (rectangle in the upper plot). Individuals from different colonies form more or less distinct groups.

We used hierarchical partitioning (Chevan and Sutherland, 1991) to estimate the importance of dissimilarity in hydrocarbon compositions, morphological distance, genetic similarity, and geographic distance between colonies for the aggression between colonies. Hierarchical partitioning is a protocol in which all

possible models in a multiple regression setting are jointly considered to identify the most likely causal factor. This is done by averaging the increase in the goodness-of-fit caused by each independent variable across all possible models (for details see Chevan and Sutherland, 1991). This averaging is likely to solve the problem of correlations among independent variables, which severely influences all one-model techniques, such as stepwise protocols (e.g., see MacNally, 2000; see also Kaib et al., 2002).

RESULTS

Chemical Analysis of Cuticular Hydrocarbons. Mean chemical distances (Nei-distance) between nestmates (major workers) were always low and ranged from 0.003 (standard deviation SD = 0.002, $N = 66$) in colony 1B1 to 0.022 (SD = 0.022, $N = 66$) in colony 1C4. In contrast, mean chemical distances between major workers from different colonies differed considerably and ranged from 0.013 (SD = 0.007, $N = 144$) between the colonies 2B1 and 1B1 to 3.566 (SD = 0.362, $N = 144$) between the colonies 1B2 and 1B3. In only two cases out of 45 (1B1–2B1, 1B1–1B2), were the intercolonial distances as low as the intracolonial differences. The principal coordinate analysis of the similarity patterns between individuals suggested four discrete hydrocarbon phenotypes (Figure 2). Within one phenotype, the variation between colonies was small. Nevertheless, individuals from one colony clustered consistently together (Figure 2).

Phenotype I (2B2, 1C4) as well as phenotype II (1B3 and 1D1) were each found at two different locations. Phenotype III was found in one colony only (1D2). Phenotype IV was most frequent and was found in five colonies (1B1, 1B2, 2B1, 2B3, 1C3) collected from three of the four focus locations. The same phenotype was also found in a colony (R1) over 20 km distant from the 10 focus colonies (this colony was not included in the analyses presented later). In each of the four focus locations, different hydrocarbon phenotypes were found.

The hydrocarbons identified ranged from C_{20} to C_{33} in chain length and were *n*-alkanes, alkenes, and alkadienes, as well as trace amounts of methylalkanes (Table 1). Chemical compositions were dominated by hydrocarbons with an uneven chain length (Table 1). The phenotypes can be chemically characterized by *n*-alkanes and alkenes, which together make up 88.8% (type I) to 98.0% (type III) of the total hydrocarbons (Table 2). *n*-Alkane compositions were largely congruent in phenotypes I and IV with a high abundance of *n*-heneicosane and *n*-tricosane. Comparing chemical phenotypes, classes of substances differ in chain length. Such a pattern in the change of chain length became apparent in the alkenes with high abundance of 5-tricosene and 11-tricosene in phenotypes I and IV, respectively. 9-Hentriacontene was the most abundant compound in phenotype II, as was 9-heptacosene in phenotype III, and both alkenes were restricted to the respective phenotype.

TABLE 1. PERCENTAGES OF THE TOTAL HYDROCARBON FRACTION IN THE CUTICLE OF MAJOR WORKERS IN THE TERMITE *Macrotermes subhyalinus*

		Type I		Type II		Type III		Type IV	
RI	Component	Mean	SE	Mean	SE	Mean	SE	Mean	SE
2060	10-Heneicosene	0	0	0	0	0	0	0.20	0.05
2100	*n*-Heneicosane	13.21	0.31	0	0	0	0	18.20	0.57
2160	10-&11-Docosene	0	0	0	0	0	0	0.62	0.09
2200	*n*-Docosane	1.67	0.03	0	0	0.03	0.03	1.20	0.05
2252	6,9-Tricosadiene	4.88	0.65	0	0	0	0	0.48	0.09
2261	11-Tricosene	3.00	0.19	0	0	0	0	39.49	0.62
2272	9-Tricosene	2.06	0.43	0	0	0	0	0	0
2284	5-Tricosene	16.06	0.41	0	0	0	0	0	0
2300	*n*-Tricosane	40.70	0.90	0	0	24.86	0.60	23.07	0.85
2336	11-&13-Methyltricosane	0	0	0	0	0	0	0.81	0.14
2351	6,9-Tetracosadiene	0	0	0	0	0	0	0.11	0.04
2400	*n*-Tetracosane	0	0	0	0	0.37	0.09	0	0
2409	Unknown	0	0	0	0	0	0	0.02	0.01
2450	Unknown	0	0	0	0	0	0	0.13	0.03
2453	6,9-Pentacosadiene	3.97	0.51	0	0	0	0	0	0
2459	11-Pentacosene	0.13	0.13	0	0	0	0	4.04	0.29
2465	9-Pentacosene	0.00	0.00	0	0	0	0	1.18	0.31
2474	7-Pentacosene	1.08	0.23	0	0	0.48	0.08	0	0
2485	5-Pentacosene	1.18	0.03	0	0	0.00	0.00	0	0
2500	*n*-Pentacosane	2.18	0.05	11.17	0.63	9.22	0.26	0.95	0.04
2513	Unknown	0	0	0	0	0	0	0.01	0.01
2566	9-Hexacosene	0	0	0	0	0.81	0.03	0	0
2661	11-Heptacosene	0	0	0	0	0.11	0.06	0.28	0.05
2669	9-Heptacosene	0	0	0	0	50.55	0.92	0	0
2676	7-Heptacosene	0	0	0	0	5.40	0.25	0	0
2700	*n*-Heptacosane	2.79	0.10	11.15	0.49	2.63	0.09	2.93	0.08
2733	11-&13-Methylheptacosane	0	0	0	0	0.95	0.05	0	0
2749	Unknown	0	0	0	0	0.38	0.14	0	0
2800	*n*-Octacosane	0.17	0.06	0	0	0.04	0.04	0.26	0.04
2868	9-Nonacosene	0	0	0.94	0.13	0.84	0.03	0	0
2877	7-Nonacosene	0	0	8.05	0.24	0	0	0	0
2900	*n*-Nonacosane	4.13	0.14	5.25	0.20	2.36	0.09	3.96	0.10
3043	8,22-Hentriacontadiene	0	0	1.53	0.12	0	0	0.01	0.01
3052	7,23-Hentriacontadiene	0	0	0.95	0.15	0	0	0	0
3069	9-Hentriacontene	0	0	25.60	0.56	0	0	0	0
3074	7-Hentriacontene	0	0	4.17	0.21	0	0	0.01	0.01
3100	*n*-Hentriacontane	0.32	0.08	2.09	0.19	0.31	0.14	0.64	0.14
3231	Tritriacontadiene	0.11	0.08	15.47	0.40	0	0	0.06	0.03
3238	Tritriacontadiene	0	0	7.27	0.25	0	0	0	0
3262	Tritriacontene	0	0	1.37	0.17	0	0	0	0

Note. The table gives the mean (±SE) across all major workers within one phenotype (Type I: $N = 18$, Type II: $N = 24$, Type III: $N = 12$, Type IV: $N = 60$). RI = retention index. The means may not sum up to exactly 100% (rounding errors).

TABLE 2. HYDROCARBON COMPOSITION IN THE CUTICLE OF MAJOR WORKERS IN THE TERMITE *M. subhyalinus*

	-ane	-ene	-di	Me-	Unknown
Total number of peaks	9	18	8	4	4
Type I	65.17	23.63	8.85	0.00	0.00
Type II	29.66	62.82	2.48	0.00	0.00
Type III	39.83	58.19	0.00	0.95	0.38
Type IV	51.21	45.89	0.61	0.81	0.15

	5-ene	7-ene	9-ene	11-ene
Type I	72.90	4.57	8.72	13.29
Type II	0.00	19.44	41.71	2.18
Type III	0.00	10.10	89.71	0.19
Type IV	0.00	0.02	2.57	95.53

Note. (Type I: $N = 18$, Type II: $N = 24$, Type III: $N = 12$, Type IV: $N = 60$). The upper part of the table gives the sum of all saturated (-ane), unsaturated (-ene, -di), as well as branched hydrocarbons (Me-) (in %). The lower part gives a more detailed analysis of the differences in alkenes (percentage of the total alkenes) between the phenotypes. Note the shift by two CH_2-groups from phenotype I to phenotype IV. The means may not sum up to exactly 100% (rounding errors).

Aggression and Hydrocarbons—A Search for Correlations. As already discussed in detail by Jmhasly and Leuthold (1999), *M. subhyalinus* shows discriminatory behavior and aggression that varies according to the specific combinations of colonies sampled within a rather small spatial scale (for the data see Table 3). In pairings of individuals from the same colony, no aggression was found, whereas in pairings from different colonies, the aggression index may range from 0 to more than 6. The observation of multiple chemical phenotypes, as well as variation in aggression among pairings of colonies, calls for an analysis of the correlation between these two data sets. However, aggression may be based on other cues than cuticular hydrocarbons, e.g., cues collected from the environment, morphological differences, or cues based on genetic differences. Thus, we include in our search of patterns, the genetic similarity between colonies (measured by the Jaccard-index from AFLP-fingerprints), the morphometric distance, as well as the geographic distance. The latter served as an indirect measure of environmental similarity.

Among colonies, mean chemical distances, the level of aggression, morphometric distances, and genetic similarity, were all correlated (Figure 3). Only geographic distance shows no correlation with any of the other parameters (Table 4). Most importantly, chemical distance correlates positively with morphological distance, and negatively with genetic similarity (Figure 3, note that this implies that genetically similar colonies have similar hydrocarbon compositions). In turn,

TABLE 3. ALL POSSIBLE COLONY PAIRINGS (45) AND ASSOCIATED MEASURES OF DIFFERENCE

Colony combinations		Pairing of phenotype	Aggression	Hydrocarbons	Morphology	Genetics	Geography (m)
1B1	2B1	IV-IV		0.01	15.9	0.683	577.2
1B1	1B2	IV-IV	0.00	0.01	14.9	0.792	16.8
2B2	1C4	I-I		0.03	16.6	0.800	387.6
1B3	1D1	II-II	0.00	0.03	120.0	0.677	268.8
2B1	2B3	IV-IV	0.00	0.03	29.4	0.677	86.4
1B2	2B1	IV-IV		0.03	12.6	0.675	562.8
2B1	1C3	IV-IV	0.40	0.04	12.7	0.679	374.4
1B1	2B3	IV-IV		0.05	35.3	0.799	518.4
2B3	1C3	IV-IV	0.02	0.06	21.2	0.737	316.8
1B1	1C3	IV-IV		0.06	11.3	0.724	206.4
1B2	2B3	IV-IV		0.06	32.2	0.799	504.0
1B2	1C3	IV-IV		0.10	10.9	0.714	192.0
2B2	2B3	I-IV	1.20	0.39	26.7	0.798	38.4
2B3	1C4	IV-I		0.42	36.8	0.819	355.2
2B2	1C3	I-IV		0.43	12.0	0.747	350.4
1C3	1C4	IV-I	0.04	0.47	13.7	0.742	38.4
2B1	2B2	IV-I	3.31	0.54	12.6	0.703	54.0
2B1	1C4	IV-I		0.58	8.4	0.687	410.4
1B1	2B2	IV-I		0.70	21.4	0.779	552.0
1B1	1C4	IV-I		0.74	10.2	0.796	170.4
1B2	2B2	IV-I		0.80	12.9	0.779	538.8
1B2	1C4	IV-I	2.17	0.90	16.1	0.783	84.0
2B2	1D2	I-III		0.95	11.6	0.757	765.6
1C4	1D2	I-III	1.59	0.96	17.6	0.791	379.2
1C3	1D2	IV-III		1.31	7.1	0.735	392.4
2B3	1D2	IV-III		1.40	12.7	0.794	734.4
2B1	1D2	IV-III		1.55	15.3	0.684	788.4
1B1	1D2	IV-III	3.21	1.77	18.4	0.777	216.0
1B2	1D2	IV-III		2.09	17.0	0.769	228.2
1D1	1D2	II-III	2.83	2.53	52.6	0.678	33.6
1B3	1D2	II-III	2.49	2.77	80.1	0.666	240.0
1C4	1D1	I-II	5.04	2.91	80.4	0.677	411.6
1B3	1C4	II-I	6.34	3.02	85.12	0.673	142.8
2B2	1D1	I-II	4.89	3.10	55.6	0.704	799.2
1B1	1D1	IV-II		3.14	96.3	0.668	249.6
1B1	1B3	IV-II	3.65	3.21	77.7	0.664	76.8
1B3	2B2	II-I		3.22	59.2	0.680	530.4
2B1	1D1	IV-II		3.23	56.0	0.674	820.8
2B3	1D1	IV-II	3.49	3.25	68.4	0.675	765.6
1B3	2B1	II-IV		3.28	71.4	0.754	550.8
1B3	2B3	II-IV	6.38	3.34	129.1	0.667	496.8
1C3	1D1	IV-II		3.35	60.6	0.697	448.8

TABLE 3. CONTINUED

Colony combinations		Pairing of phenotype	Aggression	Hydrocarbons	Morphology	Genetics	Geography (m)
1B3	1C3	II-IV		3.45	77.6	0.656	180.0
1B2	1D1	IV-II		3.54	90.6	0.655	261.6
1B2	1B3	IV-II	5.88	3.57	56.6	0.646	40.8

Note. In aggression (aggression; from Jmhasly and Leuthold, 1999), in the composition of hydrocarbons (mean Nei-distance), in morphometric dissimilarity (morphology; measurements from minor soldiers; Mahalanobis-distance), in genetic similarity (genetics, Jaccard-index from AFLP-fingerprints), and geographic distance between colonies (geography; see Figure 1). Note that the aggression data do not cover all possible combinations. Furthermore we indicate for each colony pairing also the pairing of the phenotypes (see Figure 2).

chemical distance, morphological distance, and genetic similarity are all significantly correlated with the level of aggression. However, the independent contributions (Figure 3) suggest that the difference of the hydrocarbon compositions among colonies is the data set that explains most of the variation in aggression among individuals from different colonies.

The difference in the hydrocarbon compositions among colonies as measured by the Nei-index is anonymous and provides no information about possible correlations of the difference in the relative amount of individual peaks to the aggression of pairings of colonies. Thus, we used individual peaks for a more detailed search. First of all, we found that differences in the relative amount of individual peaks never reached the predictive power of the Nei-distance ($r^2 = 0.79$; Table 4). Squared correlation coefficients exceeded 0.1 in only a few cases, and the chain lengths of these compounds were 23 and 25. Overall, we found a negative correlation between chain length and squared correlation coefficients (Figure 4). In a further step, we used multiple regression coefficients to predict the level of aggression between pairings of colonies from the differences in the relative amount of individual peaks. Using 10 compounds, we were able to explain almost 100% of the variation in aggression between colonies (Figure 5). Most of these compounds were unsaturated and/or merely minor compounds. Note that we present no formal statistical tests. This has three reasons. First, with differences in the relative amount of peaks among colonies being measured as Nei-distances, the data set is a matrix that calls for special test procedures (Manly, 1997). Second, we calculated about 40 such correlations with the risk of chance correlations, and third, the individual peaks are not independent as the sum of all peaks is always 100%.

DISCUSSION

Our results provide the following answers to the questions posed within the introduction: 1. There is sufficient variation in the hydrocarbon composition among

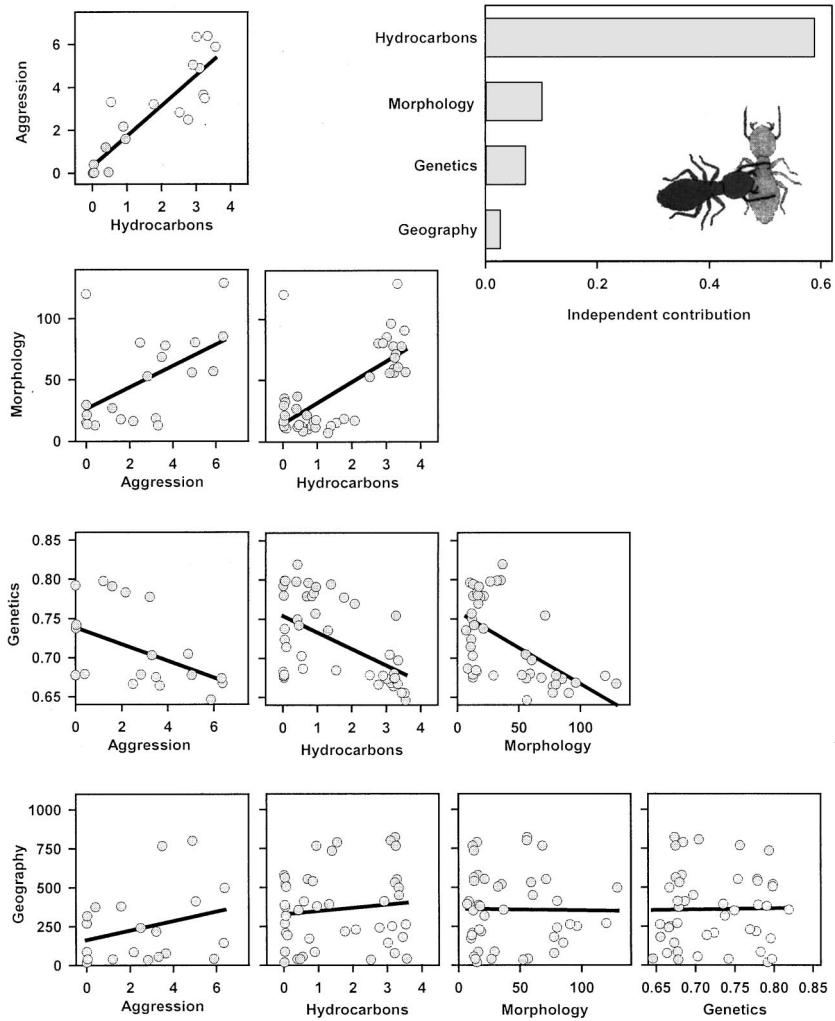

FIG. 3. All possible matrix correlations of distances or similarities among colonies (data see Table 3). The distance (or similarity) matrices measure aggression among colonies (aggression), differences in the composition of hydrocarbons (mean Nei-distance), morphometric dissimilarity among colonies (morphology; Mahalanobis-distance between colonies), genetic similarity among colonies (genetics, Jaccard-index from AFLP fingerprints), and geographic distance among colonies (geography; see Figure 1). The correlation coefficients and the associated error probabilities are tabulated in Table 4. Note the close correlation between aggression and hydrocarbons. The independent partitions also show that differences in the composition of cuticular hydrocarbons among colonies form the most important variable explaining variation in aggression between colonies.

TABLE 4. MATRIX CORRELATIONS (LOWER TRIANGLE) AND ASSOCIATED ERROR PROBABILITIES (999 PERMUTATIONS; ONE-TAILED PROBABILITIES; UPPER TRIANGLE) FOR THE DIFFERENT DISTANCE MATRICES (DATA SEE TABLE 3: PLOTS FIGURE 3)

	Aggression	Hydrocarbons	Morphology	Genetics	Geography
Aggression	—	0.002	0.008	0.038	0.10
Hydrocarbons	**0.89**	—	0.034	0.039	0.15
Morphology	**0.53**	**0.70**	—	0.017	>0.3
Genetics	**−0.46**	**−0.54**	**−0.58**	—	>0.3
Geography	0.29	0.12	−0.14	0.02	—

Note. Significant values are given in bold. The distance (or similarity) matrices measure aggression between colonies (aggression), difference in the composition of hydrocarbons (mean Nei-distance), morphometric dissimilarity between colonies (morphology; Mahalanobis-distance between colonies), genetic similarity between colonies (genetics, Jaccard-index from AFLP-fingerprints), and geographic distance between colonies (geography; see Figure 1). Note that the aggression data do not cover all possible combinations and, thus, the number of data points is 20 for all matrix correlations in which aggression is involved. All other matrix correlations are based on 45 data points (all possible combinations of 10 colonies).

colonies to allow for nestmate recognition. However, among some colonies the differences are small. 2. The level of aggression among colonies increases with an increase in the difference of cuticular hydrocarbons. Thus, individuals seem to adjust their behavior according to the differences in the composition of cuticular hydrocarbons. 3. Genetic, morphometric, and especially environmental differences, are of minor importance in predicting aggression relative to cuticular hydrocarbons.

Our analysis provides evidence that hydrocarbon compositions represent, at least in part, the cues for nestmate recognition. Furthermore, analysis of single peaks suggests that termites use a bouquet of compounds to recognize nestmates. This discrimination seems to be based not on the major components, but instead on components present only in lower concentrations and mostly unsaturated hydrocarbons. Of course, correlation does not prove causality. Thus, further experimental work should concentrate on unsaturated minor compounds in the hydrocarbon mixtures to increase the mechanistic understanding of nestmate recognition.

In the present study, variation of cuticular hydrocarbons was always low within colonies and was pronounced among colonies. Comparable chemical analyses in termites have been performed in different species of *Zootermopsis* (Haverty et al., 1988), in *Drepanotermes perniger* (Brown et al., 1996), in *Nasutitermes acajutlae* (Haverty et al., 1996c), in *Coptotermes formosanus* (Haverty et al., 1996b), and in *Macrotermes falciger* (Kaib et al., 2002). The most extensive work has been done in *Reticulitermes* (Haverty et al, 1996a, 1999; Haverty and Nelson, 1997; Page et al., 2002) and in *Heterotermes* (Watson et al., 1989). There is no reason to believe that the four chemical phenotypes we found correspond to subspecies

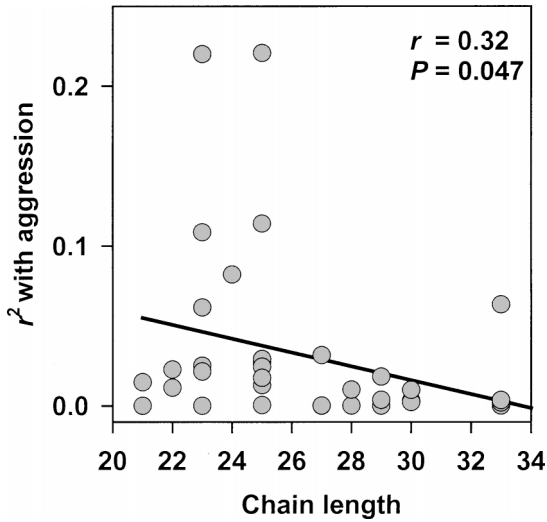

FIG. 4. Plot of squared correlation coefficients between the squared difference of individual compounds and aggression among colonies (see also insert in Figure 5) versus the chain length of the compounds. Note that most of the higher correlations ($r^2 > 0.1$) occur in compounds with a chain length of 23 and 25.

or cryptic species. No morphological, behavioral, or ecological differences were found among the 10 investigated colonies. Note also that the chemical phenotypes occur sympatrically. Haverty and Thorne (1989) distinguished two subspecies of *Zootermopsis nevadensis* on the basis of hydrocarbon phenotypes. Their investigation, however, covered the whole state of California and they did not find more than one hydrocarbon phenotype in any one specific location (see also discussion in Kaib et al., 2002).

Interestingly, the variation among colonies is not continuous. Phenotypes do not cover the whole range of possible hydrocarbon compositions, but seem to follow discrete patterns. This was also reported for *M. falciger* (Kaib et al., 2002). Chemical phenotypes differ in chain length of the hydrocarbons (elongation or shortening by one or several acetyl groups) or by a shift of the double bond along the chain, again by an even number of carbon atoms. The patterns of chain length and double bond positions in *M. subhyalinus* and *M. falciger* suggests that chain elongation or shortening of alkenes in termites may occur at the long end, as well as at the short end, of the carbon chain. These structural changes are in good agreement with the biosynthesis of insect cuticular hydrocarbons in which ethyl groups are supposed to be the "units" affected by enzymes (Blomquist et al., 1998). Hence, only few changes in the biochemical pathway are needed to generate such differences. This is a further hint that the phenotypes belong to the same species.

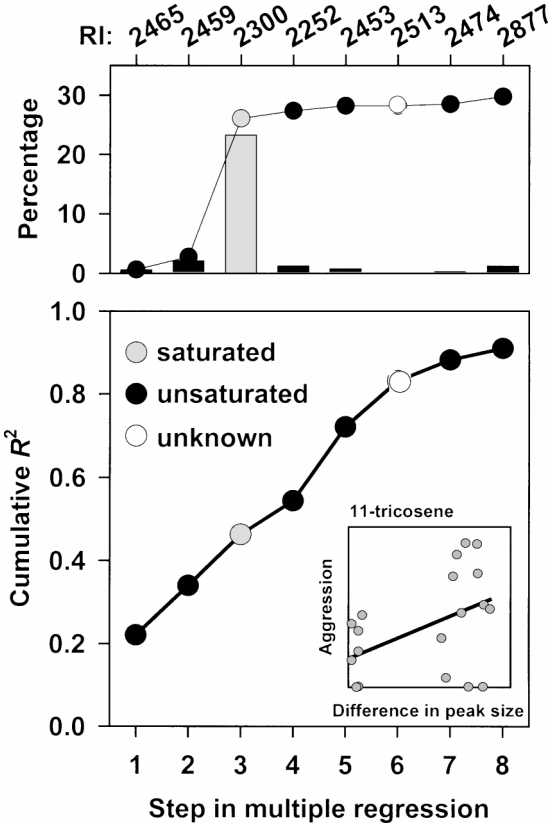

FIG. 5. Step-wise multiple correlation coefficient (R^2) of aggression among colonies versus squared differences of individual peaks. The lower graph plots the increase of the squared multiple correlation coefficient with each step. The upper graph gives the mean cumulative percentage of these peaks (%). The mean percentage of each peak was calculated as the mean across all individuals. Note that with only one exception, all selected compounds (retention indices RI according to Table 1 are given on the top of the upper graph) are only minor peaks that contribute less than 3% to the total hydrocarbon composition. The inset shows an example of the correlation of the squared difference in one selected compound versus aggression among colonies.

Also note that the genetic differences between colonies (measured by AFLPs, see Figure 3) are rather small. Colonies have between 60 and 80% of bands in common (Table 3).

In *Reticulitermes*, experiments with washed and retreated lures (Howard et al., 1982; Bagnères et al., 1991; Takahashi and Gassa, 1995) also suggested that cuticular hydrocarbons are involved in species and nestmate recognition. Furthermore,

in *Zootermopsis*, correlations between agonistic behavior and hydrocarbon phenotypes were found (Haverty and Thorne, 1989). However, these studies referred mostly to species recognition and not to intraspecific colony recognition, and the origin of labels is unknown. On the other hand, some studies in ants (Obin, 1986; Kaib et al., 1993), as well as in the termite *Coptotermes formosanus* (Su and Haverty, 1991), showed no evidence that cuticular hydrocarbons are involved in colony recognition. As alternative recognition cues, fatty acids or esters have been proposed (Heinze et al., 1996).

Altogether, convincing evidence has accumulated showing that cuticular hydrocarbons are involved in the recognition process. If we accept this, a further question arises: Are the hydrocarbons acquired from the environment, or are the differences in the hydrocarbon composition based on genetic differences? Our study favors the second possibility, but other authors provide evidence for the former (e.g., Gamboa et al., 1986). Our conclusion is based on the positive correlation of the differences in hydrocarbon composition (and of the aggression index) with morphometric differences, as well as the negative correlation with genetic similarity.

In this study, morphologic data were based on minor soldiers, whereas the other approaches used major workers. This has technical reasons. Soldiers' cuticle is highly sclerotized and does not shrink during alcohol fixation. In contrast, workers do not possess frontal gland secretion that may hamper chemical analysis or the isolation and amplification of genomic DNA. Nevertheless, the data sets from the different castes can be correlated, as in *M. subhyalinus* soldiers show the same differential response to nonnestmates as workers (Jmhasly and Leuthold, 1999). Furthermore, cuticular hydrocarbon compositions do not differ between workers and soldiers from the same colony (M. Kaib, unpublished data).

Husseneder et al. (1998) found a negative correlation between morphometric distances or the level of aggression, and genetic similarity in *Schedorhinotermes lamanianus*. Hence, in *S. lamanianus* and *M. subhyalinus* morphological differences among colonies seem to be based on genetic differences between colonies and are not the result of environmental differences faced by each colony. We sampled colonies from four different locations. However, none of the other data sets correspond with the geographic population structure (geographic distance). Thus, correlations found among chemistry, behavior, genetics, and morphology are not due to local adaptations of the colonies.

The good correlation of morphology and genetics in termites may be explained by the fact that termites live within closed colonies and under fairly constant climatic conditions, at least during their development within the colonies. Environmental variations and differences do not have as much influence on morphology as in species exposed to fluctuations of the environment during their development. The clear correlations of morphology and genetics with hydrocarbons suggest that the composition of cuticular hydrocarbons has a genetic basis

(see also Husseneder et al., 1997, 1998). A genetic basis for recognition cues was found in several species of *Hymenoptera* (see reviews in Breed and Bennett, 1987; Hölldobler and Wilson, 1990). In termites, the heritability of recognition cues was established for *Microcerotermes arboreus* (Adams, 1991). Genetically based recognition cues might facilitate the acceptance of kin and may help to increase the inclusive fitness (Gamboa et al., 1991). Alternatively, as Beye et al. (1997) note, such cues might have evolved to function as a mechanism to permit the segregation of colonies within the same environment.

Among colonies with the same chemical phenotypes, we found little or no aggression. Thus, the colony recognition system is not perfect (see Table 3). Why is the system of colony recognition imperfect? If there is a need to guarantee colony integrity, individuals should always show some aggression towards nonnestmates. There are several possible explanations. First, the experimental situation in neutral arenas might be too artificial to measure all the details of behavioral interactions among colonies. Second, colonies may use additional cues to distinguish nestmates from nonnestmates. For example, in *M. falciger*, individuals from neighboring colonies were not aggressive to each other, although they had different compositions of cuticular hydrocarbons (dear-enemy phenomenon; Kaib et al., 2002). This provides evidence that termites use additional information in their decision-making to become aggressive or not (see also Husseneder et al., 1997). In *M. subhyalinus*, there was no indication of a dear-enemy phenomenon (see Jmhasly and Leuthold, 1999). At present, we have no conclusive answer to explain the imperfect system of nestmate recognition. It may be that aggression among phenotypes is only a nonadaptive by-product of small evolutionary differences among colonies. This may have a substantial impact on other processes during the life cycle of the termites. If alates also use hydrocarbons to recognize possible partners to found a colony and hydrocarbon composition of alates is the same as of the parent colony, phenotypes would lead to assortative mating and to independent evolutionary lineages, a possibility for sympatric speciation.

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MALE AGGREGATION PHEROMONE OF DATE PALM FRUIT STALK BORER *Oryctes elegans*

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Abstract—Laboratory and field investigations were carried out to characterize the chemical communication system of the date palm fruit stalk borer, *Oryctes elegans*, and to develop pheromone-based trapping in Eastern Iran. Adults of both sexes feeding on date palm pieces attracted conspecifics, whereas date palm alone was minimally attractive. Males were twice as attractive as females. More beetles were captured at the palm crown than at ground level. Odors from adults feeding on sugarcane were sampled and analyzed by gas chromatography and mass spectrometry. Whereas females did not emit sex specific volatiles, males emitted a blend of 4-methyloctanoic acid (**1**: major component) and ethyl 4-methyloctanoate (**2**), occasionally mixed with minor components: 4-methyloctanyl acetate (**3**), methyl 4-methyloctanoate (**4**), 4-methyloctanol (**5**), and nonanyl acetate (**6**). Electroantennography and field trapping experiments demonstrated that compound **1** is an essential component of the male aggregation pheromone of *O. elegans*. It was barely attractive by itself but synergistic with fresh date palm odor. It attracted many more beetles than any of compounds **2–6**. The addition of one or several of compounds **2–6** to **1** did not improve trap captures. During the course of 2 years, we captured 4000 beetles, with a

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weekly average of 6.3 beetles/trap, and were able to monitor the seasonal flight of *O. elegans*. Our results provide the basis for developing mass trapping for control of this pest.

Key Words—Coleoptera, Scarabaeidae, *Oryctes*, aggregation pheromone, EAG, field trapping, date palm, synergy.

INTRODUCTION

Rhinoceros beetles constitute a group of medium- to large-sized scarabs of the Dynastinae subfamily whose males are characterized by a large cephalic horn. Among rhinoceros beetles, the genus *Oryctes* is specific to the Old World and includes about 40 species (Lepesme, 1947; Endrödi, 1985). Many rhinoceros beetles are associated with palm trees and are severe pests of coconut, oil, and date palms (Lepesme, 1947; Bedford, 1980). Adult beetles burrow galleries in the fresh tissues of palms for feeding. The damage is particularly severe when the apical bud is attacked in the young trees or the fruit-stalk in producing trees. Indirect damage often occurs in areas occupied by palm weevils (*Rhynchophorus* spp.), which lay eggs in the galleries made by the dynastine beetles. Palm weevil infestations are often fatal to palms. Larvae have no direct impact on plant production because most of them feed on dead tissue. Despite insecticides active against most rhinoceros beetles, efficient and acceptable methods of controlling them are still lacking in many cases, either because of economic reasons, or more generally, because adults spend most of their life hidden in galleries, and are excellent flyers, capable of rapidly colonizing new feeding and breeding sites.

The idea of manipulating adult behavior, especially luring beetles into traps, was investigated in the 1970s. Some attractants were reported but subsequently abandoned (Julia and Mariau, 1976; Vander Meer et al., 1979; Vander Meer and McGovern, 1983). Recently, male aggregation pheromones were reported from four rhinoceros beetles attacking palm trees: *Oryctes monoceros* (Olivier) in Africa (Gries et al., 1994), *Oryctes rhinoceros* L. (Hallet et al., 1995; Morin et al., 1996) in the Asia-Pacific area (further referred to as "the Tropical Oryctes"), *Scapanes australis* Boisduval in the New Guinea region, and *Strategus aloeus* L. in the New World (Rochat et al., 2000b, 2002). Synthetic pheromone makes it possible to capture these species in bucket traps. Applications on a large scale have been reported for *O. rhinoceros* and *Scapanes australis* (Hallet et al., 1995; Ho, 1996; Chung, 1997; Purba et al., 2000) but not for *O. monoceros* and *S. aloeus*. For both *O. rhinoceros* and *S. australis*, attraction to the pheromone was enhanced by, or synergistic with, plant odors from decaying wood in *O. rhinoceros* (Hallet et al., 1995; Alfiler, 1999; Sudharto et al., 2001), or from fresh palm tissues in *S. australis* (Rochat et al., 2002).

Three *Oryctes* species develop specifically in date palms and are present in the Near- and Middle-East and in North Africa: *O. elegans* Prell [=*sinaicus* Petrovitz

(non Walker)], *O. agamemnon* Burmeister (=*desertorum* Arrow, =*persicus* Endrödi), and *O. richteri* Petrovitz (Endrödi and Petrovitz, 1974). The first two are common throughout the Near- and Middle-East and seem to be sympatric in many places. They are univoltine, flying from spring to autumn. Adults cause damage by mining the stalks of fruit bunches. In the Tropical Oryctes, larvae develop in the wood of dead trees, and adults feed on living trees. The two feeding habitats are separate in space. In contrast, larvae and adults of the date palm *Oryctes* spp. live in the same living and standing trees. Larvae develop in the crown and at the periphery of the stem, feeding at the interface of dead and living tissues. Though not reported as major pests, *O. elegans* and *O. agamemnon* inflict chronic damage in date palm groves and cause economic losses in Iran and Iraq (Hurpin and Fresneau, 1969; Gharib, 1970; Hussain, 1974). Insecticide applications in the crown against emerging adults are commonly made in Iran in spring and summer. Winter pruning and burning of the dead fronds and of the dried frond bases just below the crown is recommended and applied by some growers to eliminate larvae and pupae. Both measures require considerable labor and their impact is not known.

As ethyl 4-methyloctanoate had been reported as the male aggregation pheromone of the Tropical Oryctes (Gries et al., 1994; Hallet et al., 1995; Morin et al., 1996) and also identified in a preliminary analysis of airborne effluvia from *O. elegans* males (Rochat and Malosse, unpublished), we tested this compound as an attractant for trapping *O. elegans* in Southern and Eastern Iran in 1996–97. Several weeks of trapping in highly infested areas, without using plant material as a co-attractant led to the capture of just two adults of *O. elegans* (Avand-Faghih and Rochat, unpublished). To understand this apparent contradiction and develop a trapping strategy against this insect, we undertook the study of the chemical communication of *O. elegans*. The work was based on a field approach with experimental trapping using live insects followed by screening of synthetic putative pheromone compounds selected from the results of volatile collections, gas chromatography–mass spectrometry analyses, organic synthesis, and electroantennographic screening.

Here we report the identification and activity of the male aggregation pheromone of *O. elegans* and provide practical data to develop an efficient trapping system. The result of a 6-month population monitoring study is presented, and the differences between the chemical communication systems of the rhinoceros beetles are discussed.

METHODS AND MATERIAL

Synthetic Chemicals. Chiral molecules were used as racemic mixes. 4-Methyloctanoic acid (**1**) and ethyl 4-methyloctanoate (**2**) were purchased from E.G.N.O.-Chimie (Tancarville, France) and served as starting material to prepare

4-methyloctanyl acetate (**3**), methyl 4-methyloctanoate (**4**), and 4-methyloctanol (**5**). **4** was prepared from a mixture of methanol, **1**, and sulfuric acid; **5** was obtained by reduction of **2** with $LiAlH_4$ in anhydrous diethyl ether; **3** and nonanyl acetate (**6**) were prepared from **5** and nonanol, respectively using acetic anhydride in pyridine. **1**–**6** were >98% pure by gas chromatography (GC) and **3**–**5** were free of detectable traces of **1** and **2**. Octanoic (=caprylic; **7**) and nonanoic (=pelargonic; **8**) acids were purchased from Aldrich (**7**: UK and **8**: Germany) and were 99.5 and 96% pure, respectively.

Insects. O. elegans were collected in date palm groves in the Saravan area, shipped by air to France, and maintained on sugarcane at 23–28°C, 75% RH, and under a 13L:11D photoperiod. Sugarcane (commercial source) was used as food to replace date palm core not available in France, as for other palm rhinoceros beetles (Morin et al., 1996; Rochat et al., 2000b, 2002).

Collection of the Natural Pheromone. O. elegans pheromone was collected from effluvia by passing a stream of air (purified over activated charcoal; 100 ml/min) into 1-l glass flasks containing four to six adult insects. Odors were adsorbed onto 350 mg Supelpak^{TM}-2 (SUPELCO, USA) for 4–7 d. Effluvia were trapped from batches of males, or females, or both sexes placed on two 20 cm halved sugarcane pieces, and from sugarcane controls. Five series of collections were carried out between 1996 and 2002, yielding extracts from males ($N = 7$), females ($N = 6$), and males plus females ($N = 2$) with sugarcane, and sugarcane control ($N = 4$) odors. The adsorbent was eluted with 1.5 ml 99.5% dichloromethane (1996 and 1998) or hexane (2000–2002). The extracts were stored at −40°C and used for GC and/or GC–MS.

GC–MS analyses were carried out using a Varian 3300 gas chromatograph coupled to a Nermag R10-10C quadrupole mass spectrometer. Electron impact spectra were obtained at 70 eV. Additional spectra were obtained in chemical ionization mode using NH_3 as reactant gas. The chromatograph was typically equipped with a WCOT fused silica column (30 m × 0.32 mm id, 0.5-μm MDN-5S phase; Supelco, Bellefonte, PA, USA) operated from 50°C (1 min) to 300°C at 10°C/min. Additional GC analyses were carried out using a Varian 3400 CX GC with an equivalent apolar column or a polar column with same dimensions (RTX-Wax phase; Restek, Bellefonte, PA, USA) and operated from 50°C (1 min), then heated at 15°C/min to 80°C/(9 min), then heated to 240°C at 6°C/min. Helium was used as carrier gas at 13 psi. Samples were injected using a SPI injector at 250°C. The amount of each male-specific compound was estimated by the percentage of its FID (GC) or TIC (GC–MS) response in the total response recorded to all the male-specific compounds (Table 1).

Electroantennography. Electroantennograms (EAGs) were recorded using the same equipment as described for *O. rhinoceros* (Morin et al., 1996), from dissected antennae. The antennal lamellae were held apart using small insect pins. The recording electrode was placed on the central lamella, while the reference

TABLE 1. CHARACTERISTICS AND ABUNDANCE OF THE MALE-SPECIFIC COMPOUNDS IDENTIFIED BY GAS CHROMATOGRAPHY AND MASS SPECTROMETRY FROM *Oryctes elegans* EFFLUVIA TRAPPED ON SUPELPAK™-2

Compound	Molecular weight	GC retention times (min)		EI mass spectrum at 70 eV[c] characteristic ions m/z (%)	% ratio and frequency in male effluvia[d]
		Apolar RTX-5MS[a]	Polar RTX-Wax[b]		
1: 4-Methyloctanoic acid	158	14.85–15.35[e]	29.22	41, 43, 55, 57 (100), 60, 69, 73, 83, 99, 101, 129	53 ± 11 (8)
2: Ethyl 4-methyloctanoate	186	15.24	14.70	41, 43, 45, 55, 57, 60, 61, 70, 73, 83, 88 (100), 99, 101, 123, 129, 141, 157	37 ± 10 (8)
3: 4-Methyloctanyl acetate	186	15.61	16.33	41, 43 (100), 56, 61, 69, 70, 84, 98, 101, 126	8 ± 4 (4)
4: Methyl 4-methyloctanoate	172	13.19	12.79	41, 43 (100), 55, 57, 59, 69, 74, 87, 99, 115, 123, 141, 143	1 ± 1 (3)
5: 4-Methyloctanol	144	11.85	19.07	41, 43, 45, 56, 69 (100), 70, 84, 98, 126	1 ± 1 (3)
6: Nonanyl acetate	186	17.05	17.98	41, 43 (100), 55, 56, 61, 70, 84, 98, 116, 126	0 ± 1 (2)

[a] 25 m × 0.32 mm id × 0.5 μm df (Restek) operated from 50°C for 1 min, 50–100°C at 15°C/min, 1 min at 100°C, and 100–280°C at 5°C/min.
[b] 30 m × 0.32 mm id × 0.5 μm df (Restek) operated from 50°C for 1 min, 50–80°C at 15°C/min, 9 min at 80°C, and 80–240°C at 6°C/min.
[c] Nermag R10-10C quadrupole mass spectrometer.
[d] Mean ± S.E. based on total FID or TIC response measured for the male-specific peaks. The number of occurrences is given in brackets ($n = 9$).
[e] Elutes as a broad asymmetric peak overlapping with the peaks of **2** and **3** when large amounts are injected.

electrode was pushed into the lumen of the scape. Each antenna was bathed by a flow of moist air (400 ml/min) and stimulated with compounds **1**–**4** and **7**–**8**. Each compound was delivered at four doses, selected randomly from serial dilutions in hexane (0.1–100 μg; decadic step) The antenna was stimulated by hexane (solvent) at the beginning, the end, and between each dose series. Compounds were presented as 1 μl of hexane solutions deposited on filter papers inserted in Pasteur pipettes just prior to the stimulation (0.5 sec; 200 ml/min). Raw EAG values for a given antenna were normalized to relative EAGs (percent) by dividing them by the average response recorded to the complete stimulation series.

Field Trapping Experiments: Chemical Dispensers. Synthetic chemicals were emitted from heat-sealed sachets made of polyethylene films (ALPLAST, France), the characteristics and dimensions of which were adapted to the chemicals and the targeted release rates after laboratory calibration (e.g., 28 $\times$ 40 $\times$ 0.2 mm filled with 0.7 ml to emit **1** at 3 mg/d at 35°C). Calibration was achieved by preparing dispensers of various sizes and different film thicknesses, filled with the pure chemicals, and left in a room thermostated at 30, 35, and 40°C for one wk at each temperature. The dispensers were weighed daily, and the slopes obtained from the linear adjustment of the weight loss versus time furnished the estimations of the evaporation rates. The dispensers were stored at -10°C and weighed (mg precision) prior to and at the end of each replicate or assay to determine the daily release rates of the chemicals (reported in the figures and tables).

Location and General Features. All assays were carried out close to the Pakistan border in Saravan or Zaboli, a place about 100 km west from Saravan. Both areas are located in Sistan and Balootchestan province of Iran and constitute the eastern limit of the area of date palm production in Iran, with a mean elevation of ca. 1200 m above sea level and having cold winters. The areas consist of scattered oases, with a traditional farming system of mixed vegetables, pasture, and fruit crops, with few or no chemical inputs, either fertilizer or pesticide. Date palms are mostly Mazafati variety, in highly heterogeneous plots.

Traps consisted of 24-l plastic buckets whose lids were equipped with eight radial 8 $\times$ 5 cm openings. All treatments (except Assay 8) included one piece of date palm core (ca. 1 kg) freshly cut from a shoot. The palm piece was wrapped in a punctured plastic bag to limit dehydration (synthetic lures) or put in a 1-l perforated plastic box with two live *O. elegans*. In Assay 8, date palm core tested without beetle was prepared as described above or by chopping the palm piece and crushing the tissue with a hammer.

In Assays 1–2, traps were put on the ground at the base of a date palm. From Assay 3 onwards, traps were hung from the stem at 2.5–3 m above ground level, generally positioned just below the crown of the palms. Soapy water was poured into the bottom of the bucket to prevent beetles from escaping. No insecticide was used. Assays 1–8 were carried out in randomized complete block designs. Traps were spaced $\geq$80 m apart. The two blocks were separated by $\geq$200 m. One assay

consisted of *n* consecutive periods designated as replicates. During each replicate, the date palm pieces remained unchanged and the traps were visited at regular intervals (every 3 or 7 d) to collect trapped beetles, check the health of the *O. elegans* used as bait, and replace dead bait insects and empty chemical dispensers as needed. Date palm tissue was renewed and the treatments were re-randomized at each replicate (Table 2).

Chronology and Experimental Choices. The experiments are summarized in Table 2. Host plant was systematically included as bait (except specific Assay 8) because compound **2** alone had captured almost no beetles. The attraction to conspecifics was investigated first. Then we searched for the best trap positioning (Assays 1–3). Synthetic chemicals were further evaluated as pheromone candidates in various situations (Assays 4–8). Finally, Assay 9 evaluated the feasibility of monitoring *O. elegans* population over one flight season.

Statistical Analysis. (Minitab, 1998). Assays 4–8: The total captures per trap per visit were analyzed by a three-way ANOVA (F1: bait; F2: age of date palm tissue–no. of visit within replicate; F3: replicate) on $\mathrm{Ln}(x + 1)$ transformed data using a general linear model (GLM) procedure. Means were compared by multiple comparison (Tukey test) or comparison to a reference (Dunnett test) with $P = 0.05$. The data from the four assays were pooled and subjected to another common ANOVA to evaluate the effect of the age of the date palm tissue. In assays 1–3, we could not apply ANOVA to the raw data because too many values were zero. We performed an ANOVA on cumulative captures per trap: the total per replicate and the mean daily total per assay for Assays 3 and 1–2, respectively. Results of Assays 1–2 were analyzed together because both had the same structure and gave similar results. The sex ratio of the captures was analyzed similarly in all assays. Details of these analyses are not presented because the sex ratio appeared not to be correlated to the experimental variables and to be variable across the trapping periods (e.g., Figure 1).

RESULTS

Effluvia Characteristics and Pheromone Structural Identification. Feeding by beetles of either sex induced the emission of various molecules (f; Figure 1A). Comparisons of the odors emitted by only sugarcane, and males, females, or both sexes feeding on sugarcane showed that the presence of males was correlated with at least six volatile molecules occurring in variable proportions (Figure 1; Table 1). In contrast, no female-produced chemicals were detected. Based on their mass spectra and retention times by GC, and comparisons with reference compounds, the male-specific molecules were identified as **1**: 4-methyloctanoic acid, **2**: ethyl 4-methyloctanoate, **3**: 4-methyloctanyl acetate, **4**: methyl 4-methyloctanoate, **5**: 4-methyloctanol, and **6**: nonanyl acetate (Table 1). At least one of these compounds

TABLE 2. GENERAL CHARACTERISTICS OF NINE FIELD EXPERIMENTS CARRIED OUT TO TRAP *Oryctes elegans* USING NATURAL OR SYNTHETIC ATTRACTANTS IN EASTERN IRAN FROM 1998 TO 2002 WITH REFERENCE TO THE FIGURES AND TABLES SHOWING THE RESULTS

Expt. #	Purpose (no. treatments) *Baits* (added to date palm core except Assay 8)	Characteristics: blocks, no. × duration of replicate (frequency of visits)[a]	Date and place
1	Attraction by conspecifics and sex effect (4) *Baits*: 2♂, 2♀, 2♂+2♀, no beetles (c)	4 blocks, 4 × 14 d (7 d) Zaboli	May 21–Jul 20, 1998, Zaboli
2	Attraction by conspecifics and sex effect (4) *Baits*: 2♂, 2♀, 2♂+2♀, no beetles (c)	4 blocks, 10 × 9 d (3 d)	May 5–Aug 3, 1999, Saravan
3	Effect of trap height from ground (9) *Baits*: 2♂ at 0, 2, 5 m, 2♀ at 0, 2, 5 m, no beetles at 0, 2, 5 m *Synthetic compounds (male-specific: 1–6 or analogs: 7–8)*	4 blocks, 2 × 9 d (3 d)	Jul 9–Sep 10, 2000, Saravan
4	Test of compounds **1**–**3** emitted by same dispenser model (6) *Baits*: **1**, **2**, **3**, **1**:**2** (1:1 v/v), **1**:**2**:**3** (1:1:1), 2♂ (c)	5 blocks, 4 × 9 d (3 d)	Jun 14–Jul 20, 2001, Saravan
5	Test of compounds **1**-**8** at same target dose of 5 mg/day (9) *Baits*: **1**, **2**, **3**, **4**, **5**, **6**, **7**, **8**, 2♂ (c)	4 blocks, 2 × 9 d (3 d)	Jun 20-Jul 8, 2002, Saravan
6	Effect of adding compounds **2**–**6** to compound **1**: test of five **1**–**6** mixes (B1–B5) at target dose of 5 mg/day vs. only **1** (9) *Baits*: B1, B2, B3, B4, B5, **1** (3 and 6 mg/day target doses), 2♂ (c)	5 blocks, 2 × 9 d (3 d)	Jul 26-Aug 13, 2002, Saravan
7	Dose-response to compound **1** (5) *Baits*: four doses of **1**, 2♂ (c)	6 blocks, 2 × 9 d (3 d)	Jul 8–26, 2002, Saravan
8	Synergy between compound **1** and date palm core -DP- (5) *Baits*: **1**, DP, crushed DP, **1**+DP, **1**+crushed DP, 2♂+DP (c)	5 blocks, 2 × 9 d (3 d)	Aug 13–31, 2002, Saravan
9	Seasonal population monitoring (1) *Bait*: compound **1** (target dose of 3 mg/d)	10 traps, 25 × 7 d (7 d) Saravan	Apr 7–Sep 1, 2002, Saravan

Note. (c): control.

[a] For example: Assay 3 (four complete blocks) lasted 4 × 9 d = 36 d. The number of visits (basic data for ANOVA) was 4 × (9 d/3 d) = 12. In Assay 8, the 10 traps (single treatment) were visited weekly for 25 wk.

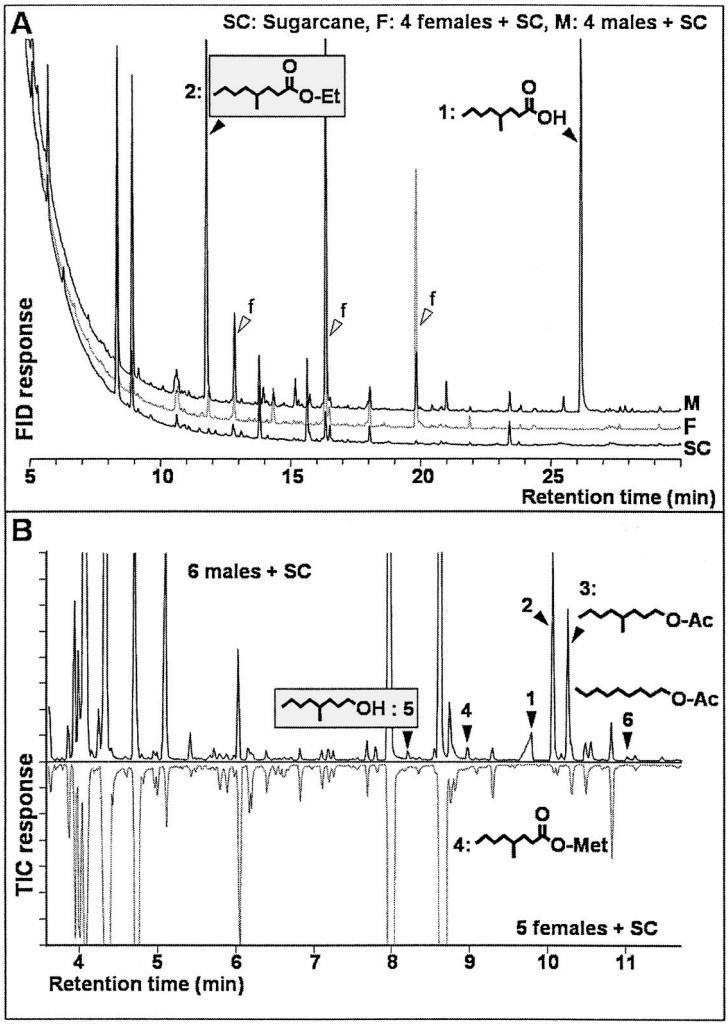

FIG. 1. Overlaid chromatograms of Supelpak^{TM} -2-trapped volatiles emitted by sugarcane, and *Oryctes elegans* males or females feeding on sugarcane, showing two profiles of male-associated compounds. A: GC analysis on a polar RTX-Wax column showing male effluvia characterized by **1** and **2** (55:45 ratio; 2001 collection) and food volatiles induced by the beetle feeding (f: white arrow heads); and B: GC–MS analyses using an apolar MDN-5MS column showing compounds **1– 6** (21:37:34:3:4:1 ratio) associated with males (2000 collection).

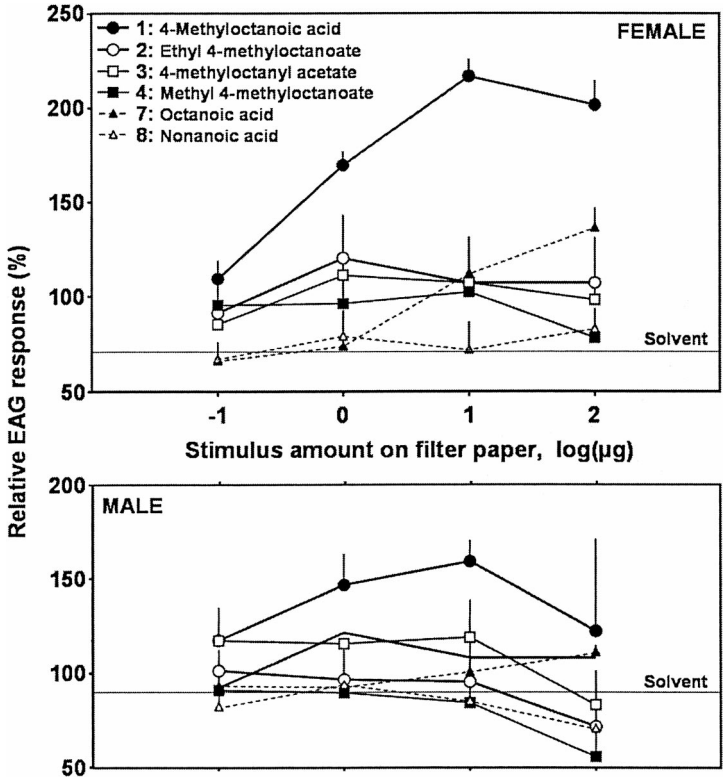

FIG. 2. Relative electroantennogram (EAG) responses by *Oryctes elegans* males and females (mean + S.E.; $N = 4$ of each sex) to six synthetic molecules (four compounds isolated from the male effluvia: **1–4**; and two related molecules: **7–8**) at four doses. The horizontal lines within the graphs indicate the mean responses to solvent.

was detected from eight of the nine batches with males. **1** and **2** were the most abundant and regular components (respectively, 53 ± 11 and $37 \pm 10\%$ total male-specific volatiles; mean $\pm$ S.E.). **1** was emitted at a daily rate estimated between 0.1 and 5 μg/male. In six batches, **1** was more abundant than **2** (80 to 50%) followed by **2**. In two cases, **2** was the most abundant male-specific molecule (88 and 37%) either mixed with only **2** (12%) or **3** (35%) and other minor components. **3–6** were detected in less than half the samples. **3** was the third most abundant component ($8 \pm 4\%$) whereas **4–6** appeared as sporadic trace components, accounting for $<1\%$ of the male-specific volatiles.

Electroantennography. (Figure 2). EAG responses recorded to the six tested molecules differed between sexes. In females, most of the responses to the chemicals were higher than to the solvent. The most stimulatory compound was the

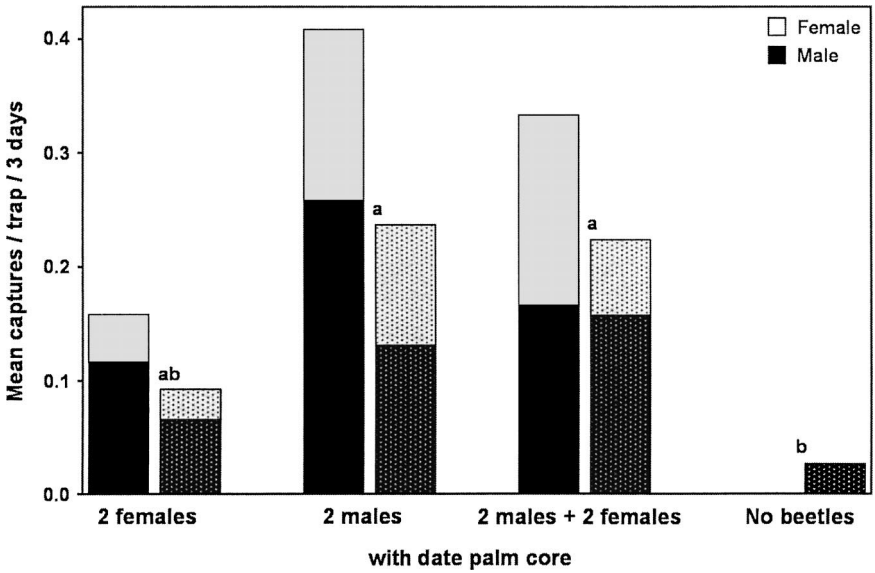

FIG. 3. Captures of *Oryctes elegans* by bucket traps baited with a piece of date palm core with or without caged live conspecifics in two experiments in Eastern Iran [Right dotted bars: Experiment 1, total captures = 44 (1♂:0.5♀); left plain bars: Experiment 2, total captures = 108 (1♂:0.7♀)]. Captures surmounted by thy same letter are not statistically different in both experiments (Tukey multiple comparison test, $P < 0.05$).

acid **1**, which triggered responses that increased with dose. Compound **8** elicited negligible responses, and compound **7** elicited only small responses. The other male-specific compounds (**2**–**4**) did not elicit clear EAGs. In males, the responses were less strong and only responses to **1** were greater than to the solvent. The highest dosage of most of the molecules appeared to inhibit responses.

Trapping with Live Beetles. In experiments 1–2, using live beetles as bait, few beetles were caught. Nevertheless the effect of the bait was significant on the captures ($F(3, 31) = 7.91$, $P < 0.001$; Figure 3). The traps with male *O. elegans* bait (with or without females) captured more conspecifics than the traps without beetles, (Tukey test; $P < 0.05$; Figure 3). The traps with females as bait captured an intermediary number of beetles, about half of those containing only two males (with or without females). The difference was suggestive but not significant ($P = 0.07$ and $P = 0.18$ for female vs. male and male + female, respectively) due to high variability and a small number of replicates (4).

In experiment 3, the height of the traps influenced the captures whatever the bait ($F(2, 215) = 4.14$, $P < 0.017$) with higher traps being more effective. Traps placed about 5 m above ground captured more beetles than traps on the ground.

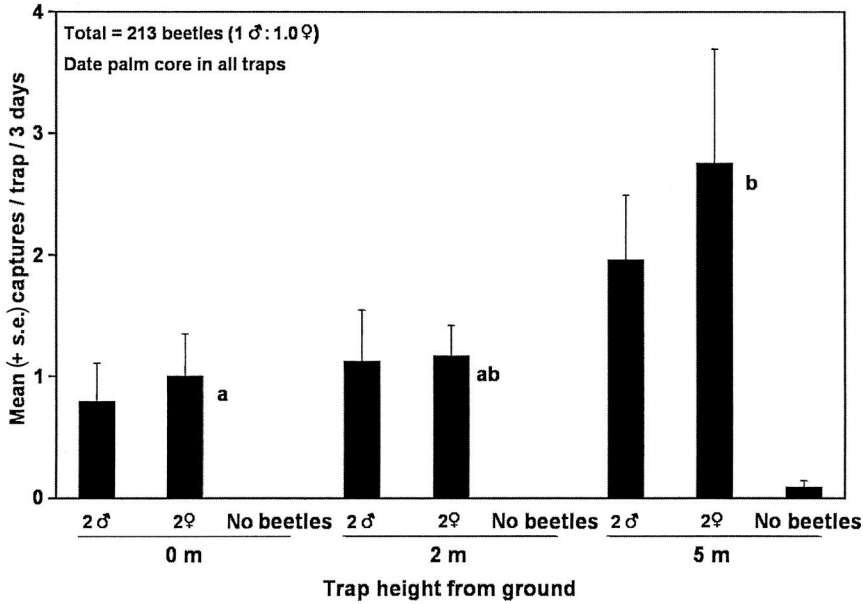

FIG. 4. Effect of the position of the trap on captures of *Oryctes elegans* using a piece of date palm core with or without caged live conspecifics as bait (Eastern Iran Assay 3). Whatever the bait, captures associated with the same letter are not statistically different. Catches by males and females were statistically equivalent and greater than by no beetles (Tukey multiple comparison test, $P < 0.05$).

At about 2 m high, traps captured an intermediate number of insects. Attraction to odors of males and females was equivalent and greater than to traps with no beetles whatever the trap position (Tukey test, $P < 0.05$; Figure 4).

Evaluation of Synthetic Chemicals. Whatever the synthetic bait and the replicate, trap catches decreased as the date palm tissue aged ($F(2, 1115) = 67.23$, $P < 0.001$; Figure 5) in experiments 4–8. The mean captures were significantly lower at a given visit than at the previous one. They decreased by 20 and 66% respectively at the second (day 6) or third (day 9) visit as compared to the first (day 3).

The effect of the replicate (time) was significant in experiments 4, 5, and 7, ($F(3, 354) = 40.23$, $F(1, 208) = 43.32$, and $F(1, 179) = 5.23$, respectively; $P < 0.001$), indicating that the level of catches changed with time whatever the baits. In experiments 6 and 8, the level of capture was equivalent in the two replicates.

In experiment 4 (Table 3), the effect of the bait was significant ($F(5, 354) = 7.56$, $P < 0.001$). Combined with date palm core, compound **1** was most attractive, significantly more than compounds **2**, **3**, and the **2**:**3** blend (1:1). The **1**:**2**:**3** mix

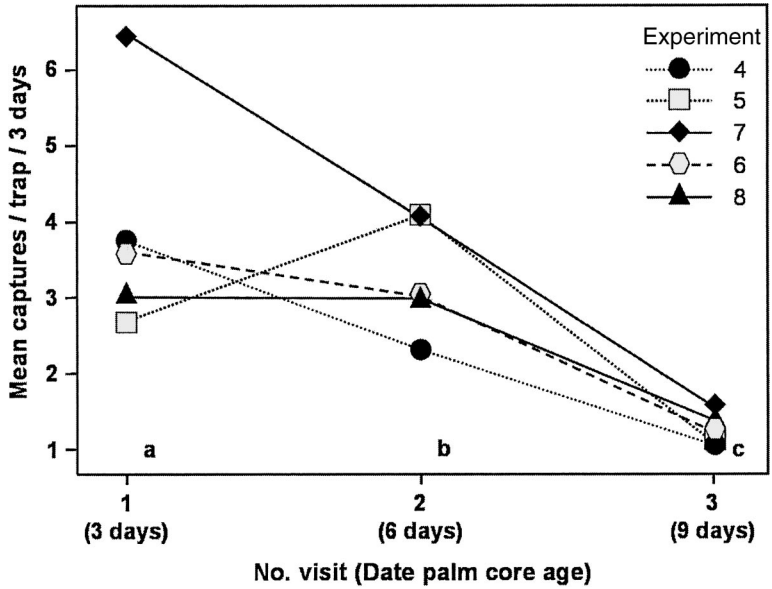

FIG. 5. Effect of date palm core aging (chronology of the visits) on the mean captures of *Oryctes elegans* by traps baited with various synthetic male-produced compounds or related compounds, or two live conspecific males, plus a piece of date palm core in Eastern Iran; common analysis on Experiments 4–8. Captures associated with different letters are statistically different (Tukey multiple comparison test, $P < 0.05$).

(1:1:1) and two live males were intermediate in attractiveness (Tukey test; $P < 0.05$). The synthetic baits were emitted within a broad range of release rates, from 2.8 (**1**) to 16.5 mg/d (**2**).

In experiment 5 (Table 4), combined with date palm core, **1** attracted larger numbers of beetles than any other treatment, including two live males. The synthetic chemicals were emitted in a narrower range than in experiment 4, from 4.3 (**1**) to 12.5 mg/d (**7**), especially five of the six compounds produced by males (4.3–6.2 mg/d).

In experiment 6 (Table 5), combined with date palm core, **1** emitted at 5.1 mg/d (reference) or 7.6 mg/d was equally attractive. The synthetic baits were emitted between 5.1 mg/d (**1**) and 8.6 mg/d (B1). There was no evidence of synergism or additive effects from the minor components. In fact, several of the blends were less attractive than **1** as a single component.

In a dose response trial (experiment 7) **1** emitted at 2.2, 3.2, or 9.2 mg/d, combined with date palm core was equally attractive. A release rate of 1 mg/d was less attractive, as was the trap baited with two male beetles (+date palm core). (Dunnett test; $P < 0.05$.)

TABLE 3. COMPARATIVE CAPTURES OF *Oryctes elegans* BY THREE SYNTHETIC COMPOUNDS (**1–3**: THE MAJOR COMPONENTS OF THE MALE EFFLUVIA), ALONE OR MIXED, AND EMITTED BY THE SAME TYPE OF DISPENSER, AND TWO LIVE CONSPECIFIC MALES IN BUCKET TRAPS CONTAINING A PIECE OF DATE PALM CORE (EASTERN IRAN, EXPERIMENT 4)

Bait added to date palm core[a]	Mean ± S.E. release rate of the chemical bait (mg/day)[b]	Mean ± S.E. captures/trap/3 d[c]
1: 4-Methyloctanoic acid	2.8 ± 0.4	4.0 ± 0.6^{b}
2: Ethyl 4-methyloctanoate	16.5 ± 1.3	1.4 ± 0.2^{a}
3: 4-Methyloctanyl acetate	13.6 ± 0.3	2.2 ± 0.5^{a}
2:**3** (1:1)	15.6 ± 1.0	1.5 ± 0.3^{a}
1:**2**:**3** (1:1:1)	8.5 ± 0.4	2.6 ± 0.5^{ab}
Two males (control)	—	2.5 ± 0.5^{ab}

Notes. Total catches = 844 (1♂ : 1.4♀). S.E.: standard error of the mean.
[a] Values in brackets indicate the relative proportion of the components within mixes.
[b] Actual rates during the experiment ($n = 25$). Values for each component of the mixes undetermined.
[c] Captures followed by the same letter are not significantly different (Tukey multiple comparison test, $P < 0.05$).

In the 8th experiment (Table 6), captures by all the baits were significantly different from zero. Crushed date palm core tended to attract more beetles, either alone (×2.6) or combined with **1** (×1.4), than a standard date palm core piece. As before, **1** combined with date palm core was more attractive than two live males on date palm core, alone, or date palm core alone (Dunnett test; $P < 0.05$.)

TABLE 4. COMPARATIVE CAPTURES OF *Oryctes elegans* BY EIGHT SYNTHETIC MOLECULES AT THE SAME TARGET DOSE OF 5 MG/DAY (SIX COMPOUNDS ISOLATED FROM THE MALE EFFLUVIA: **1–6** AND TWO RELATED MOLECULES: 7–8), AND TWO LIVE CONSPECIFIC MALES IN BUCKET TRAPS CONTAINING A PIECE OF DATE PALM CORE (EASTERN IRAN, EXPERIMENT 5)

Bait added to date palm core	Mean ± S.E. release rate of the chemical bait (mg/day)[a]	Mean ± S.E. captures/trap/3 d[b]
1: 4-Methyloctanoic acid	4.3 ± 0.2	9.6 ± 2.5^{a}
2: Ethyl 4-methyloctanoate	5.5 ± 0.2	2.3 ± 1.0^{b}
3: 4-Methyloctanyl acetate	8.2 ± 0.1	2.7 ± 0.3^{b}
4: Methyl 4-methyloctanoate	4.8 ± 0.2	0.9 ± 0.9^{b}
5: 4-Methyloctanol	6.2 ± 0.4	2.7 ± 0.8^{b}
6: Nonanyl acetate	5.4 ± 0.2	0.5 ± 0.7^{b}
7: Octanoic acid	12.7 ± 2.0	1.0 ± 0.2^{b}
8: Nonanoic acid	9.5 ± 0.5	1.7 ± 0.6^{b}
Two males (control)	—	1.8 ± 0.6^{b}

Notes: Total catches = 653 (1♂ : 0.5♀). S.E.: standard error of the mean.
[a] Actual rates during the experiment ($6 \leq n \leq 8$).
[b] Captures followed by the same letter are not significantly different (Tukey multiple comparison test, $P < 0.05$).

TABLE 5. COMPARATIVE CAPTURES OF *Oryctes elegans* BY FIVE SYNTHETIC MIXES (B1-B5) OF THE SIX COMPOUNDS ISOLATED FROM THE MALE EFFLUVIA **1–6**, TWO DOSES OF **1,** THE MAJOR COMPOUND OF THE MALE EFFLUVIA, AND TWO LIVE CONSPECIFIC MALES IN BUCKET TRAPS CONTAINING A PIECE OF DATE PALM CORE IN EASTERN IRAN (EXPERIMENT 6)

Bait added to date palm core[a]	Mean ± S.E. release rate of the chemical bait (mg/day)[b]	Mean ± S.E. captures/ trap/3 d[c]
B1: **1**:**2**:**3** (1:1:1)	8.6 ± 1.2	1.8 ± 0.4*
B2: **1**:**2**:**3** (10:1:1)	5.2 ± 0.1	2.0 ± 0.3*
B3: **1**:**2**:**3**:**4**:**5**:**6** (100:10:10:1:10:1)	5.3 ± 0.3	3.3 ± 0.6
B4: **1**:**2**:**3**:**4**:**5**:**6** (10:10:10:1:10:1)	7.3 ± 0.8	3.3 ± 0.6
B5: **1**:**2**:**3**:**4**:**5**:**6** (10:10:10:1:1:1)	7.6 ± 0.8	1.5 ± 0.3*
1: 4-Methyloctanoic acid	5.1 ± 0.2	→3.9 ± 0.6
1: 4-Methyloctanoic acid	7.6 ± 0.5	4.5 ± 0.7
Two males (control)	—	0.7 ± 0.2*

Notes. Total catches = 623 (1♂ : 1.3♀). S.E.: standard error of the mean.
[a] **2**: Ethyl 4-methyloctanoate, **3**: 4-methyloctanyl acetate, **4**: methyl 4-methyloctanoate, **5**: 4-methyloctanol, and **6**: nonanyl acetate). Values in brackets indicate the relative proportion of the compounds within the mixes B1–B5.
[b] Actual rates during the experiment ($9 \leq n \leq 10$). Values for each component of the mixes undetermined.
[c] Captures asterisked are significantly lower than compound **1** at 5.1 mg/d (Dunnett test, $P < 0.05$).

Population Monitoring over 25 wk (experiment 9: Figure 6). A total of 841 beetles (55% female) was captured in 10 traps. Weekly captures per trap, which were nil in mid-April, increased regularly until the beginning of June (5.3 ± 1.4 beetles). Then, they oscillated around that level until mid-September with a sharp

TABLE 6. SYNERGISTIC CAPTURES OF *Oryctes elegans* BY COMBINED DATE PALM CORE (CRUSHED OR NOT) AND SYNTHETIC **1**, THE MAJOR COMPONENT OF THE MALE AGGREGATION PHEROMONE AS COMPARED TO TWO LIVE MALE CONSPECIFICS ON DATE PALM CORE IN BUCKET TRAPS (EASTERN IRAN EXPERIMENT 8)

Bait	Mean ± S.E. release rate of the chemical bait (mg/day)[a]	Mean ± S.E. captures/ trap/3 d[b]
1: 4-Methyloctanoic acid	3.0 ± 0.1	0.3 ± 0.1[b]
Date palm core	—	0.2 ± 0.1[b]
Crushed date palm core	—	0.6 ± 0.3[b]
1 + Date palm core	3.5 ± 0.1	5.2 ± 0.7[a]
1 + Crushed date palm core	3.3 ± 0.1	7.4 ± 0.9[a]
Two males + date palm core	—	1.0 ± 0.3[b]

Notes. Total catches = 435 (1♂ : 1.5♀). S.E.: standard error of the mean.
[a] Actual rates during the experiment ($n = 8$).
[b] Captures followed by the same letter are not significantly different (Tukey multiple comparison test, $P < 0.05$).

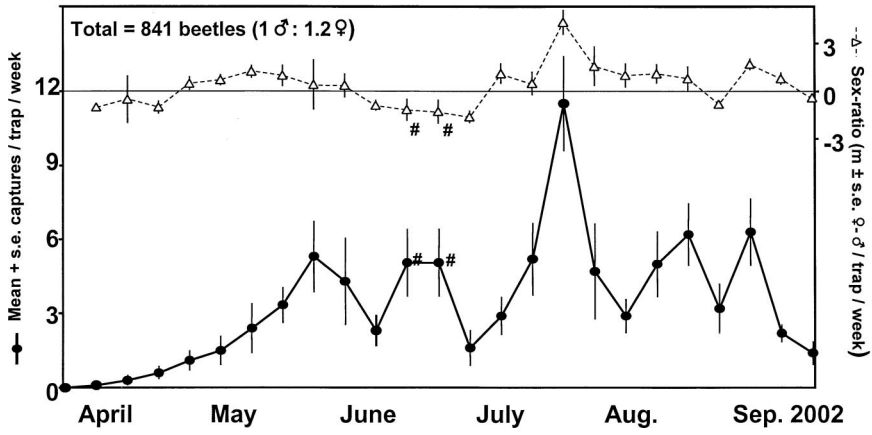

FIG. 6. Weekly captures of *Oryctes elegans* per trap—bottom, left scale—with corresponding sex-ratios—top, right scale—from 10 traps baited with a piece of date palm core and **1** (4-methyloctanoic acid**,** the major component of the male aggregation pheromone) emitted at a rate of 2.2 ± 0.1 mg/d (mean ± S.E.) for 25 wk between April and September 2002 in Eastern Iran (Experiment 9). Marked points indicate that the represented values are averages due to a single visit on July 7 with captures from 2 wk accumulated in traps.

peak of capture at the beginning of August (11.5 ± 1.9 beetles) and decreased during the two final weeks of the trial. Considering the mean captures per trap calculated on a monthly basis, the population reached a maximum in summer (mid-July–mid-August: 5.9 ± 0.8 beetles). The sex ratio of the catches fluctuated throughout the trial, being approximately balanced in spring and then showing an excess of females through summer, especially in August.

DISCUSSION

Our data demonstrate that male *O. elegans* produce an aggregation pheromone like all the dynastid species studied to date. Its active component is 4-methyloctanoic acid, a molecule previously reported from the Tropical *Oryctes*, *O. monoceros* and *O. rhinoceros* (Gries et al., 1994; Hallet et al., 1995; Morin et al., 1996). However, besides these common features, the *O. elegans* pheromone communication system differs from the pheromone communication system of the Tropical *Oryctes* in two major aspects: (1) *O. elegans'* most active pheromone compound is not active in the Tropical *Oryctes*, and (2) *O. elegans* pheromone is weakly attractive by itself but highly synergistic with the odor emitted by freshly cut and macerated palm tissue. This is not the case for the aggregation pheromones of the Tropical *Oryctes*, which are highly attractive without plant chemicals.

Attraction to Conspecifics in Natural Conditions. Both sexes of *O. elegans* attracted conspecifics when placed on date palm core, suggesting the emission of (a) pheromone(s) and/or plant volatiles induced by beetle feeding. The latter hypothesis is in agreement with the catches by either sex feeding on palm tissue recorded in the initial assays. GC analyses also showed that feeding of beetles of either sex on sugarcane resulted in the emission of several compounds.

Trap captures in traps baited with males were never significantly greater than in traps baited with females and the initial field assays did not conclusively support the emission of a male aggregation pheromone. Evidence for a male-produced pheromone was provided by the analyses of the beetles' effluvia and subsequent assays of synthetic chemicals. Conversely, the existence of a female aggregation pheromone seems unlikely because no female-specific compounds were detected from the female effluvia. Thus, attraction of beetles to females feeding on date palm may only reflect a response to palm volatiles induced by beetle feeding. In addition, our first assays were performed in harsh environmental conditions ($>50^{\circ}$C, very low humidity). These conditions were fatal to many bait insects as shown by the high mortality recorded on the weekly visits (experiments 1–2), and males probably did not emit pheromone during the major part of the assays.

Male Pheromone Composition. The male pheromone secretion of *O. elegans* was more complex and variable than reported in the Tropical *Oryctes*. In addition to the major compounds **1** and **2** that were reported from them, *O. elegans* males produced the new compounds **3**–**5**, which have obvious structural relationship with **1** and **2**, and with ethyl 3-methylheptanoate, reported from *O. rhinoceros* (Hallet et al., 1995). These molecules share a methyl-branched carbon skeleton, suggesting a common biogenetic pathway. The trace amounts of **3**–**6** detected from certain batches of male *O. elegans* could be artifacts of our sampling procedure due to reactions between fermentation products from sugarcane with compounds **1**–**2**. However, SPME sampling showed that two males placed in silanized glass vials without food could emit 0.1–1 μg of **1**, **2**, and **5** simultaneously (Rochat and Pézier, unpublished). The noticeable amounts of the acetate **3** detected in at least two batches of male effluvia also support production of **3** and **5** by the insect itself.

O. elegans male-specific effluvia were variable both in the quantities emitted and in their qualitative composition. This is possibly related to our sampling conditions because we used wild beetles, of unknown age and physiological status, at different seasons, and grouped in different numbers with only males or mixed sexes. Pheromone collection from more standardized samples, coupled to an appropriate method for GC quantification will be required to determine the biological factors which modulate pheromone emission.

Pheromone Activity. Insects responded to racemic **1**, indicating that chirality was not a critical point in the species or suggesting that it uses a racemic pheromone. Hallet et al. (1995) reported 4*S*-**2** to be as attractive as (±)-**2** in *O. rhinoceros* and concluded that this species produces either the S enantiomer or a racemic mix. We

did not investigate the enantiomeric composition of the molecules produced by *O. elegans*.

A strong response to the acid **1** was the constant feature of the comparative trials performed, in accordance with the prevalence of this molecule in the male pheromone secretion. The specificity of the response was correlated to the methyl-branched carbon skeleton because responses to the acid analogs **7** and **8** were negligible. In the field, 1–10 mg/d release rates of **1** were active. Higher doses were not evaluated because release rates of 2–3 mg/d were as good as the higher dose of 10 mg/d.

All the other male-specific chemicals were less active than **1**, either as single compounds or blends (experiment 3), and even tested at higher doses. These observations are in agreement with the strong EAG responses recorded to **1**, in contrast to those recorded to the other molecules. The presence of large amounts of **2** (ethyl ester) or **3** (acetate) with **1** (experiments 4–5) seemed to reduce the trapping efficiency with the exception of the mix **1**:**2**:**3**:**4**:**5**:**6** (10:10:10:1: 1:1) in experiment 5. The good activity of this mix may be due to the large amount of **5**, a compound that was not tested by EAG. However, because no clear effect, either inhibitory or enhancing, was observed to the blends, and because most minor male-specific chemicals appeared as erratic components in the male effluvia, the responses to the molecules other than **1**–**2** in the field may be due to their structural relationship with **1**. Alternately, these compounds might be involved in reproductive isolation with *O. agamemnon*, which is sympatric with *O. elegans* in lowland areas of the Middle East; in that case it would not be expected that their presence would improve the efficiency of the traps.

The strong activity of **1** in *O. elegans* and the weak activity of **2** is opposite to what happens in the Tropical *Oryctes*: **2** proved to be the only or essential active pheromone compound in these species while **1** resulted in negligible captures of *O. rhinoceros* at 30 mg/d (Hallet et al., 1995). **1**, inactive when evaluated by GC-EAD of natural male effluvia, was not evaluated in the field with *O. monoceros* (Gries et al., 1994). In *O. rhinoceros*, a third molecule, ethyl 3-methylheptanoate was also reported from males and attracted about four times fewer beetles than **2** at 30 mg/d (Hallet et al., 1995), indicating the species was moderately responsive to another molecule produced by males, with a close structural relationship to the major pheromone compound.

The sex ratio of the beetles captured was not correlated to the pheromone bait but varied during the flight period of the beetle. An equal ratio of males and females was captured over the whole set of experiments with some variations, the origin of which is unknown. Both sexes seemed to emerge simultaneously in spring according to the 2002 monitoring. The female excess recorded in summer may reflect a shorter life span of the males or a greater mobility of females.

Plant Co-Attraction. Although **1** as a single component and date palm odor were attractive alone catches dramatically increased when both odors were

combined. The effect was strong with fresh palm material, becoming weaker as the tissue aged, and the effect tended to be enhanced when the palm tissue was crushed. Whereas this phenomenon is classical in palm weevils (Giblin-Davis et al., 1996; Rochat et al., 2000a), it has not been reported in other dynastid beetles, or, if so, to a much lesser extent. It may be correlated to the ecology of *O. elegans*, which colonizes live trees for mating and egg laying in the same way as the palm weevils.

In the Tropical *Oryctes*, mating sites are separate from feeding sites. Males are always found singly in feeding galleries, an observation fully in agreement with the absence of pheromone emission from galleries (Morin et al., unpublished). Their aggregation pheromone is attractive by itself (Hallet et al. 1995; Morin and Allou, unpublished). An enhanced or synergistic response, not as dramatic as in *O. elegans*, can be recorded by adding decaying woody material to the pheromone of *O. rhinoceros*, an egg laying substrate where the beetles hide and the males emit their pheromone (Hallet et al., 1995; Alfiler, 1999; Sudharto, 2001).

The dynast *S. australis* presents an intermediate case between *O. elegans* and the Tropical *Oryctes*. Rochat et al. (2000b) showed that the male aggregation pheromone was quite attractive by itself but also synergistic with odors emitted by freshly cut coconut or sugarcane tissues. Male *S. australis* emit their aggregation pheromone from the feeding galleries burrowed in such fresh tissue, attracting females and mating with them, but also attracting other males which they get rid of by fighting (Prior et al., 2000). The egg-laying sites are separate from the feeding sites, as in the Tropical *Oryctes* and contrary to *O. elegans*. Therefore, a synergy between the male pheromone and plant odors appears particularly intense when it involves fresh tissues where the males are emitting pheromone. The mechanism of the plant–insect interaction, especially in the synergistic response and in the pheromone emission, remains unknown.

Applied Perspectives. More than 4000 *O. elegans* were caught over the two trapping seasons during which the synthetic pheromone was evaluated. The captures averaged 6.3 beetles/trap/wk considering only the traps baited with **1** and date palm, which emerged as the operational bait for further trapping. The 25-wk trapping campaign showed that seasonal monitoring of the pest can be carried out efficiently by this means. The trap catch data agreed with data available in the literature and obtained from farmers: *O. elegans* adults appear in spring and can be found until the temperature drops in late fall. The maximum abundance is recorded in summer, based on the individuals observed at lights.

Trapping could be used as a monitoring and decision tool to improve the timing of insecticide application, which is generally performed when farmers prune leaves around fruit bunches and place them in dried leaf nets in order to protect them from wind and predators. Insecticide is applied as a prophylatic measure, and there is no simple way to evaluate the population level. Trapping could also be integrated with insecticide applications to directly control the pest, with a double benefit: a more efficient and safer elimination of the beetles, which can be done using soapy

water without insecticide in traps as reported for *S. australis* (Rochat et al., 2002). This method is particularly attractive considering the low economic level of the farmer, the human health risks from insecticides, and the fragile environment of the oases.

Besides the cost of the pheromone, one critical point of this trapping strategy remains, i.e., the necessity to use fresh date palm tissue to synergize the pheromone. Date palm tissue must be renewed weekly in order to maintain a satisfactory level of captures. However, such material is precious for farmers because shoots are sold for the multiplication of the palms. We need to find cheap substitutes for the date palm shoots, either natural or synthetic, before trapping can be fully developed.

Finally, the development of a common trapping of *O. elegans* and the red palm weevil, *Rhynchophorus ferrugineus* Oliv., now sympatric with the dynast in many places, is also planned for a better and more economic general control of date palm beetle pests.

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ANALYSIS OF ANAL SECRETIONS FROM PHLAEOTHRIPINE THRIPS

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Abstract—The anal secretions of 16 phlaeothripine thrips species (Thysanoptera: Phlaeothripidae) were studied, including a reinvestigation of three species previously reported. A total of 37 components were detected, including hydrocarbons, acetates, terpenes, carboxylic acids, a quinone, an aromatic compound, and a pyranone compound. The secretions of all species were composed of some of these components, with *Xylaplothrips inquilinus* possessing as many as 11 components. Of these components, (*Z*)-9-octadecene, (*Z*)-9-nonadecene, nonadecadiene, octanoic acid, decanoic acid, geranial, neral, α-pinene, β-pinene, caryophyllene, 2-hydroxy-6-methylbenzaldehyde, and two unidentified monoterpenes [UK-I (M^+136) and UK-II (M^+168)] were detected for the first time. The chemicals were species-specific; four *Liothrips* species and three *Holothrips* species could be distinguished from each other and their congeners by the GC profiles of the ether extracts of their anal secretions. The anal secretions of gall-inducing thrips commonly contained terpenes, of which citral (a mixture of geranial and neral) and β-acaridial repelled ants or had antifungal activity. The findings suggest that these terpenes play a defensive role and prevent galls from fungal infestation. 3-Butanoyl-4-hydroxy-6-methyl-2*H*-pyran-2-one, found from three *Holothrips* spp., caused paralysis in ants. Chemical analysis of anal secretion components is a useful method for the classification of

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tubuliferan species that are difficult to distinguish on the basis of morphological characters.

Key Words—Phlaeothripinae, Phlaeothripidae, Thysanoptera, anal secretion, Tubulifera, thrips.

INTRODUCTION

The insect order Thysanoptera is classified into two suborders, Terebrantia and Tubulifera. Females in Terebrantia are characterized by sawlike ovipositors, not found in tubuliferan females, whereas tubuliferan species have an elongated tube (abdominal segment X). Thus, the suborders are readily distinguished morphologically. Tubulifera has only one family, Phlaeothripidae, which is further divided into two subfamilies, Phlaeothripinae and Idolothripinae. The latter is specialized for spore-feeding, and comprises about 600 species, whereas the former is a large subfamily comprising about 2500 species (Mound, 1997). Phlaeothripines are generally smaller in size than the idolothripines.

In previous papers, we reported that the anal secretions of 10 phlaeothripine species are composed of a wide variety of compounds, including hydrocarbons, acetates, terpenes, carboxylic acids, quinones, and a pyranone compound (Suzuki et al., 1986, 1988, 1989, 1993, 1995; Haga et al., 1989, 1990), whereas the 17 idolothripine species analyzed secreted only juglone and carboxylic acids (Suzuki et al., 1990, 2000). Here, we report a subsequent study that analyzed the anal secretion components of 16 phaleothripine species, including a reevaluation of three species previously reported.

METHODS AND MATERIALS

Thrips. Sixteen species of Phlaeothripinae, including the three species reinvestigated (*Liothrips kuwanai*, *L. piperinus*, and *Gynaikothrips ficorum*), were collected from host plants in the field. The species, collection site and time, host plants, and the numbers of adult thrips extracted are summarized in Table 1.

Extraction of Anal Secretions. Anal secretions were obtained by extracting insects with redistilled diethyl ether (0.5 ml) for 5 min (Suzuki et al., 1986, 2000). From our experience, anal secretions are best obtained by this routine ether extraction method. However, Porapak Q trapping (Suzuki et al., 1990) and the filter paper absorbing method were also used to confirm whether the components analyzed were derived from an anal secretion. The ether extracts were concentrated to about 0.2 ml with a N_2 stream when necessary, and 1 μl of the extract was injected into the gas chromatographs.

Chemical Analysis of Extracts. Extracts were analyzed by GC, GC-MS, and TLC, using methods described previously (Suzuki et al., 1995). GC analyses were carried out using an Hewlett-Packard 5890 II gas chromatograph, equipped with a

TABLE 1. PHLAEOTHRIPINE SPECIES ANALYZED

Species	Collected place	Month and year of collection	Host plant	Numbers extracted
Apelaunothrips consimilis (Ananthakrishnan)	Yonaguni Isl., Okinawa Pref.	April 1999	Dead foliage of a broadleaf tree	77
Dolichothrips sp.	Yonaguni Isl., Okinawa Pref.	April, 1999	*Mallotus japonicus*	81
Ecacanthothrips inarmatus Kurosawa	Hanazono, Kita-ibaraki, Ibaraki Pref.	Aug. 1990	Dead foliage of a broadleaf tree	15
Eugynothrips intorquens (Karny)	Amami Isl., Kagoshima Pref.	Feb. 1991	*Silax china* (gall)	215
Gynaikothrips ficorum (Marchal)	On campus of Univ. Ryukyu, Okinawa Pref.	Nov. 1988	*Ficus retusa* (gall)	48
Haplothrips aculeatus (Fabricius)	On campus of Univ. Tsukuba, Ibaraki Pref.	Sept. 1990	*Trifolium pretense*	351
H. kurdjumovi Karny	On campus of Univ. Tsukuba, Ibaraki Pref.	Sept. 1989	*Setaris taberi* and *S. viridis* var. *minor*	150
Holothrips yuasai (Kurosawa)	Mt. Tsukuba, Tsukuba, Ibaraki Pref.	March 1994	Dead wood	25
Liothrips kuwanai (Moulton)	Awa-kominato, Chiba Pref.	Jan. 1992	*Piper kazura* (gall)	36
L. piperinus Priesner	Awa-kominato, Chiba Pref.	Jan. 1992	*Piper kazura* (gall)	145
L. wasabniae Haga et Okajima	Izumo-shi, Shimane Pref.	Aug. 1990	*Eutrena* (*Wasabia*) *japonica*	50
L. sp. (undescribed species)	Sugadaira, Nagano Pref.	June 1992	*Vibrum opulus* var. *calrescence*	29
Mychiothrips fruticola Haga et Okajima	On campus of Univ. Tsukuba, Ibaraki Pref.	June 1990	By beating of *Pleioblastus chino*	41
Oidanothrips frontalis (Bagnall)	Ushiku-shi, Ibaraki Pref.	Oct. 1987	Dead wood	218
Psalidothrips lewisi (Bagnall)	Mt. Omoto, Ishigaki Isl., Okinawa Pref.	March 1997	Dead foliage of *Custanopsis cuspidata*	82
Xylaplothrips inquilinus (Priesner)	On campus of Ryukyu Univ., Okinawa Pref.	April 1999	*Ficus retusa* (gall)	23

fused silica CP-Sil 19 CB capillary column, and a Hitachi 263-30 gas chromatograph, equipped with a fused silica FFAP capillary column (both columns, 25 m × 0.25 mm, 0.2 μm film), using nitrogen as carrier gas. GC-MS analyses were executed with an Hitachi M-80B GC-mass spectrometer, equipped with a fused silica column (25 m × 0.25 mm; 0.1 μm film), coated with Al-clad methylsilicone, at 70 eV. Helium was used as the carrier gas. The temperature was programmed to increase 4°C/min from 90 to 200°C.

TLC was performed using silica gel 60 F_{254} plates (E. Merck, 0.20-mm thickness), with hexane–ether (8:2) as the developing solvent. Double bonds of unsaturated compounds were assigned by GC-MS analyses of dimethyl disulfide (DMDS) adducts (Francis and Veland, 1981; Vincenti et al., 1987). The geometry of mono ene compounds was determined by GC by comparison with authentic samples. For analyses of the recently collected species (*Xylaplothrips inquilinus*, *Dolichothrips* sp., and *Apelaunothrips consimilis*), samples were introduced via a split/splitless injector in the splitless mode (sampling time 1.0 min) at 250°C. The temperature was programmed to increase 8°C/min from 30 (1 min) to 250°C or 6°C/min from 30 (1 min) to 230°C for a CP-Sil or FFAP capillary column, respectively. An HP-5MS capillary column (30 m × 0.25 mm; 0.25 μm), fitted in an HP-6890 MSD, was used for GC-MS analyses. The temperature was programmed to increase 10°C/min from 30 (1 min) to 270°C. Helium was used as the carrier gas.

Chemicals. Citral (a mixture of geranial and neral), β-myrcene, juglone, and plumbagin (2-methyljuglone) were purchased from Aldrich Chemical Co. (*Z*)-3-Decenoic acid was obtained from Tokyo Kasei Co., Ltd. The preparation of perillene, β-acaridial, 7-pentadecene, 8-heptadecene, alcohol acetates, 4-decenoic acid, 3- and 5-dodecenoic acids, 5-tetradecenoic acid, 3-butanoyl-4-hydroxy-6-methyl-2*H*-pyran-2-one, and (*Z*,*Z*)-5,8-tetra decadienoic acid were as reported previously (Suzuki et al., 1986, 1988, 1989, 1990, 1993, 1995, 2000; Haga et al., 1989). 9-Octadecene and 9-nonadecene were synthesized by the Wittig reaction of appropriate phosphonium bromides with 1-decanal in the presence of NaH in DMSO.

Repellent Activity of the Secretion Components Against Ants. Secretion components were tentatively bioassayed against ants, *Formica japonica*, as previously reported (Suzuki et al., 1995), at doses of 0.1, 1.0, and 10.0 μg (10, 100, and 1000 ppm ether solutions) of the test sample. One half of a filter paper (21 mm diam) was treated with 10 μl of each sample solution, and the other half was treated with 10 μl of ether as a control. After evaporation of the solvent, the paper was placed in a Petri dish (22 mm id. × 8 mm in height), with the inside wall coated with paraffin. Five ants were released into the dish, and the dish was covered with a similar dish. The number of ants on each area of the filter paper was recorded after 5, 10, 15, and 30 min. The percent repellency was calculated separately for each replicate ($N = 5$) as follows: Repellency (%) $= 100\,(C - T)\,(C + T)$, where

C and T are the total number of ants at each time on the control and treated areas. Data were statistically analyzed using the χ^2 test.

RESULTS

A total of 37 components were found from 16 phlaeothripine species, including the three species previously reported and reinvestigated here (1–11 components per species). Thrips species and their secretion components found in the present and previous studies are listed in Table 2, according to the classification of tribes by Priesner (1961). Table 2 summarizes a total of 42 secretion components from the 23 species studied to date. Each species from all the studies cited in Table 2 contained at least one of the components. The components included hydrocarbons, acetates, terpenes, carboxylic acids, quinones, an aromatic compound, and a pyranone compound.

Liothrips kuwanai and *L. piperinus.* The secretion components of *L. kuwanai*, which induces leaf galls on the host plant *Piper kadzura*, were reported previously (Suzuki et al., 1989). Later, we noticed that the galls induced by *L. kuwanai* involved the closely related inquilinous thrips, *L. piperinus*. Thus, we attempted to reanalyze the secretions of the two *Liothrips* species. Adults were separated by observation of the middle and hind tibial color (white in *L. kuwanai*, black in *L. piperinus*), using a binocular microscope, yielding 36 *L. kuwanai* and 145 *L. piperinus* individuals. The GC profiles of the extracts were similar (Figure 1), but (*Z*)-9-hexadecenyl acetate (**17**; 8.9%) was found only in *L. kuwanai*, and the (*Z*)-7-pentadecene (**5**) content was 1.1% in *L. piperinus* and 14.2% in *L. kuwanai*. Thus, these two species were chemotaxonomically distinguished from each other by their GC patterns. The peak areas of perillene (**28**) and β-acaridial (**29**) in the gas chromatogram of the extract from *L. piperinus* (one thrips equivalent) corresponded to 1.8 and 1.0 μg, respectively, by GC comparison with synthetic perillene.

Liothrips wasabiae and *L.*. sp. The GC patterns of the extracts of these species (Figure 1) resembled that of *L. piperinus*. β-Acaridial **29** was a prominent component in *L. wasabiae* (40.1%) but a minor component in *L*. sp. (13.3%). (*Z*)-9-Hexadecenylacetate **17** was detected from *L. wasabiae* (2.7%) but not from *L*. sp. In contrast, (*Z*)-7-pentadecene **5** was found from *L*. sp. (trace amount) but not from *L. wasabiae*. These two species are not gall inducers, but they are gregarious (Haga and Tsutsumi, unpublished observations).

Gynaikothrips ficorum. The secretion components of *Gynaikothrips uzeli*, collected from leaf galls of the host plant, *Ficus retusa*, on the Ryukyu University campus, Okinawa Prefecture in November 1988, were reported previously (Suzuki et al., 1989). More recently, we reassigned this species to *G. ficorum*, which is distributed worldwide, including Japan, whereas *G. uzeli* is found only in Southeast

TABLE 2. ANAL SECRETION COMPONENTS OF PHLAEOTHRIPINE SPECIES

Species	Component[a]
Phlaeothripinae	
Tribe Haplothripini	
Dolichothrips sp.	(9), 30 (t), 33 (m), 34, 36, 37
Haplothrips aculeatus	22 (m), 25, (26)
H. kurdjumovi	20 (m), 21
Xylaplothrips inquilinus	2, 3 (t), 4, 6 (t), (9), 15 (t), 16, 18, 28, 35 (m; M^+168), 42
Tribe Hoplothripini	
Eugynothrips intorquens	8, 9, 11, 12 (m), 13, 27 (M^+ 136), 31, 32
Gynaikothrips ficorum[b]	2 (t), 3 (t), 4 (m), 8, 9, 15 (t), 16, 18 (t), 29
Hoplothrips japonicus[c]	22 (m), 23
Liothrips kuwanai[b]	2, 4 (m), 5, 9, 15, 16, 17, 28, 29
L. piperinus[b]	1 (t), 2 (m), 3 (t), 4, 5, 9 (t), 15, 16, 28, 29
L. wasabiae	2, 4, 15, 16, 17, 18 (t), 28, 29 (m)
L. sp.	2 (m), 3 (t), 4, 5 (t), 9, 15, 16, 28, 29
Ponticulothrips diospyrosi[c]	25 (m), (26), 37, 38, 39, 40 (M^+ 224)
P. sp.[c]	25, 37, 38, 39 (m), 40 (M^+ 224)
Thlibothrips isunoki[c]	30 (only)
Tribe Leeuweniini	
Varshneyia pasanii[c]	1 (t), 2 (m), 3 (t), 4, 9 (t), 15, 16, 19, 28, 29
Tribe Phlaeothripini	
Ecacanthothrips inarmatus	22 (m), 23, 24
Mychiothrips fruticola	25, 26 (m)
Psalidothrips lewisi	22 (m), 23
Tribe Docessissophothripini	
Holothrips hagai[c]	2, 3, 4 (m), 9, 10, 41
H. japonicus[c]	2, 3, 4 (m), 5, 9, 10 (m), 41
H. yuasai	2 (t), 3 (t), 4, 5, 8, 9, 10 (m), 41
Oidanothrips frontalis	4 (m), 6 (t), 8, 9, 10, 28
Tribe Apelaunothripini	
Apelaunothrips consimilis	(7), (9), 14, 16, (22; m), (23)

Note. t, trace component; m, major component; double bond location of compound (number) in parentheses not determined.

[a] Hydrocarbons—1: dodecane: 2: tridecane; 3: tetradecane; 4: pentadecane; 5: (*Z*)-7-pentadecene; 6: hexadecane; 7: hexadecene; 8: heptadecane; 9: (*Z*)-8-heptadecene; 10: heptadecadiene; 11: (*Z*)-9-octadecene; 12: (*Z*)-9-nonadecene; 13: nonadecadiene.
Acetates—14: dodecadienyl acetate; 15: tetradecyl acetate; 16: hexadecyl acetate; 17: (*Z*)-9-hexadecenyl acetate; 18: octadecyl acetate Carboxylic acids—19: 2-methylbutyric acid; 20: octanoic acid; 21: decanoic acid; 22: (*E*)-3-dodecenoic acid; 23: (*Z*)-5-dodecenoic acid; 24: dodecadienoic acid; 25: (Z)-5-tetradecenoic acid; 26: (5,8)-tetradeca-dienoic acid.
Terpenes—27: UK-I (M^+ 136), 28: perillene, 29: β-acaridial; 30: β-myrcene; 31: geranial; 32: neral; 33: α-pinene; 34: β-pinene; 35: UK-II (M^+ 168), 36: caryophyllene.
Quinones—37: plumbagin; 38: 7-methyljuglone.
Others—39: UK-III; 40: UK-IV (M^+ 224); 41: 3-butanoyl-4-hydroxy- 6-methyl-2*H*-pyran-2-one; 42: 2-hydroxy-6-methylbenzaldehyde (HMBA).

[b] Reinvestigated species.

[c] previously reported species.

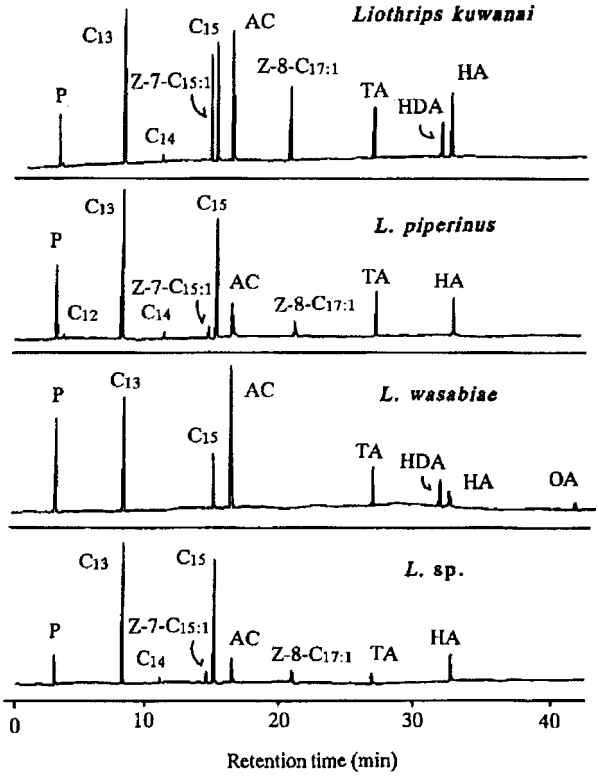

FIG. 1. GC profiles of the extracts from four *Liothrips* species. Condition: CP-Sil 19 CB, 90–200°C, 4°C/min. P: perillene; AC: β-acaridial; TA: tetradecyl acetate; HDA: (*Z*)-9-hexadecenyl acetate; HA: hexadecyl acetate; OA: octadecyl acetate.

Asia. These species are difficult to distinguish morphologically, and *G. ficorum* is possibly a variant of the type species, *G. uzeli* (Mound, 1994, 1996; Mound et al., 1996; Mound and Marullo, 1996). The secretion components are also shown in Table 2.

Eugynothrips intorquens. The gas chromatogram showed at least seven peaks (Figure 2); the largest was further separated on the polar FFAP column into two peaks, characterized as nonadecene (M^+ 266) and nonadecadiene (M^+ 264) by GC-MS analysis. Similarly, the other hydrocarbons were identified as heptadecene, heptadecane (**8**; 2.7%), and octadecene. The mono ene hydrocarbons were identified as (*Z*)-8-heptadecene (**9**; 9.8%), (*Z*)-9-octadecene (**11**; 1.4%), and (*Z*)-9-nonadecene (**12**; 38.9%) by GC-MS analysis of the DMDS adducts and direct GC comparisons with synthetic samples. The small amount of nonadecadiene (**13**; 5.4%) prevented the location of the double bonds. Geranial (**31**; 32.3%) and neral

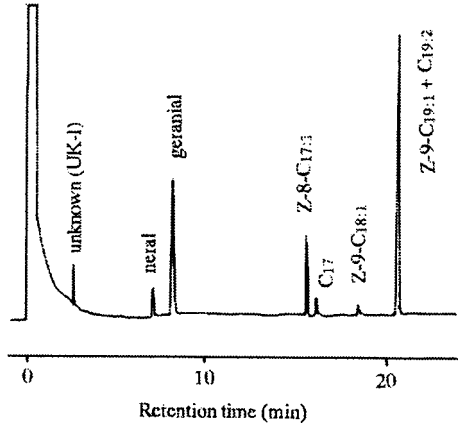

FIG. 2. Gas chromatogram of the extract of *Eugynothrips intorquens*. GC conditions: see Figure 1.

(**32**; 4.1%) were also identified by GC, GC-MS, and TLC analyses. The unknown peak [**27**; 4.0%, UK-I; M^+ 136, 82 (base peak), 67, 55, 41, 27] was thought to be a monoterpene because of its M^+ion and fragmentation pattern.

Acid emitting thrips, *Haplothrips aculeatus, H. kurdjumovi, Ecacanthothrips inarmatus, Mychiothrips fruticola,* and *Psalidothrips lewisi.* These five species were characterized by anal secretions that contained acids only, similar to those of *Hoplothrips japonicus* (Haga et al., 1989). Two tailing peaks observed in GC from *H. kurdjumovi* were tentatively identified as carboxylic acids, because their peaks were shifted by methylation with diazomethane. GC-MS and GC analyses of the methylated components revealed that they were methyl octanoate and methyl decanoate. Therefore, these acids were identified as octanoic acid (**20**; 67.4%) and decanoic acid (**21**; 22.6%). Porapak Q trapping confirmed that these acids were secretion components (Suzuki et al., 1990). Similarly, (*E*)-3-dodecenoic acid (**22**; 74.7%), (*Z*)-5-tetradecenoic acid (**25**; 11.7%), and tetradecadienoic acid (**26**; 13.6%) were found in *H. aculeatus*. *M. fruticola* emitted only two acids, 5,8-tetradecadienoic acid (**26**; 64.1%) and **25** (35.9%), which are widely found in idolothripine thrips (Suzuki et al., 1990, 2000; Blum, 1991). The secretion components of *Psalidothrips lewisi* were a mixture of (*E*)-3- (**22**; 68.5%) and (*Z*)-5-dodecenoic acids (**23**; 31.5%), similar to those of *Hoplothrips japonicus* (Haga et al., 1989).

Holothrips yuasai and *Oidanothrips frontalis.* These two species belong to the tribe Docessissophothripini, but had different secretion components. The GC profile of *H. yuasai* (Figure 3) was similar to those of *Holothrips japonicus* and *H. hagai* (Suzuki et al., 1993), particularly *H. japonicus*. The pyranone compound (**41**; 9.0%) was identified together with hydrocarbons. *H. yuasai* contained pentadecane

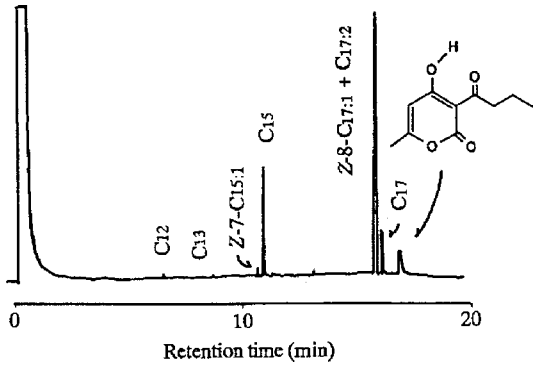

FIG. 3. Gas chromatogram of the extract of *Holothrips yuasai*. GC conditions: see Figure 1.

(**4**; 26.4%), heptadecadiene (**10**; 35.1%), and (*Z*)-8-heptadecane **9** (19.9%). Heptadecane (**8**; 8.7%) was found only from *H. yuasai* among three *Holothrips* spp. Thus, these three *Holothrips* species were chemotaxonomically distinguished by the GC profiles of their secretions. Pentadecane **4** (43.5%), perillene **28** (5.3%), and other hydrocarbons were found in *O. frontalis*.

Dolichothrips sp. and *Xylaplothrips inquilinus.* The extract of *D*. sp. was a yellowish color. A yellow component (M^+188, base peak) was identified as plumbagin (**37**; 10.4%), which was first found from *Ponticulothrips diospyrosi* and *P*. sp. (Suzuki et al., 1995). Furthermore, some mono- and sesqui terpene hydrocarbons were detected including α-pinene (**33**; 39.1%), β-pinene (**34**; 23.9%), β-myrcene (**30**; trace), and caryophyllene (**36**; 20.1%), along with heptadecene (**9**; trace), but there were no acids. *Xylaplothrips inquilinus* yielded at least 11 GC peaks (Figure 4). Peak 1 (M^+136) was identified by comparison with a synthetic standard (Leal et al., 1988) as 2-hydroxy-6-methylbenzaldehyde (HMBA) (**42**; 15.1%), which was first isolated from the acarid mite, *Acarus siro* (Curtis et al., 1981). The main component (Peak 2; 40.8%) was an unidentified monoterpenoid (UK-II) [**35**; M^+168; 150, 137, 125, 82, 81, 69 (base peak), 55, 43, 41] with an isopentenyl group, judging from the base peak at m/z 69. The MS spectrum was somewhat similar to that of perillene (**28)**. Compound **28** (5.0%), hydrocarbons, and acetates were also detected.

Apelaunothrips consimilis. In split injection mode, the extract produced only small peaks, whereas seven peaks were observed in splitless mode. Two tailing peaks were identified as dodecenoic acids because fragments at m/z 74 and 212 (M^+) appeared after methylation with diazomethane. These acids were thought to be (*E*)-3-dodecenoic acid **22** (76.4%; main component) and (*Z*)-5-dodecenoic acid **23** (3.5%) by GC comparison, but DMDS treatment was not performed because of the small amount of secretion. Hexadecene (**7**; 2.0%), heptadecene (**8**; 1.0%;

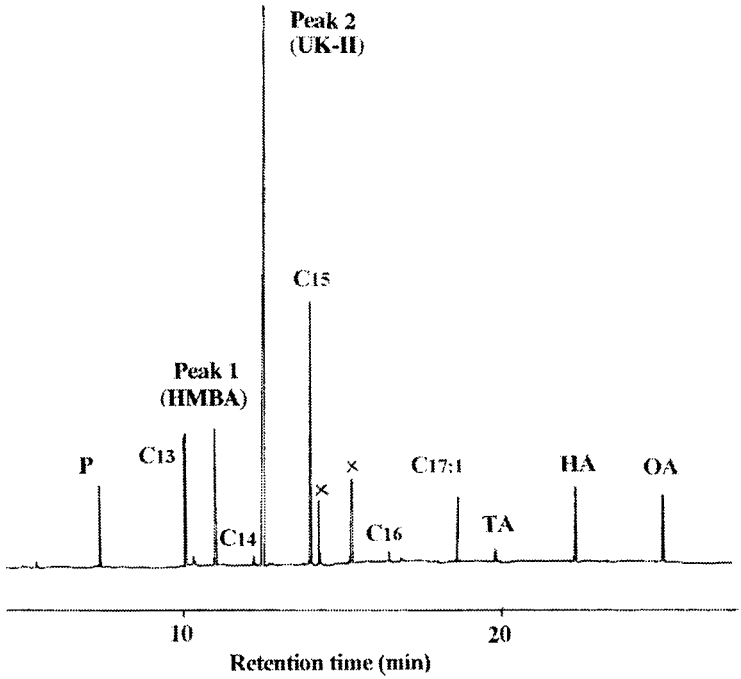

FIG. 4. Gas chromatogram of the extract of *Xylaplothrips inquilinus*. GC condition: CP-Sil 19 CB, 30 (1)-250°C, 8°C/min (splitless mode). HMBA: 2-hydroxy-6-methylbenzaldehyde; P: perillene; TA: tetradecyl acetate; OA: Octadecyl acetate.

could be (*Z*)-8-), dodecadienyl acetate (**14**; 11.4%), and hexadecyl acetate (**16**; 5.6%) were also detected, but terpenes were not detected.

Repellency of the Test Compounds Against Ants. Both citral (a mixture of geranial **31** and neral **32**) and 3-butanoyl-4-hydroxy-6-methyl-2*H*-pyran-2-one (**41**) showed significant repellency against ants at doses of 10.0 μg (Table 3). The latter compound displayed no repellency at a dose of 1.0 μg, but caused paralysis at more than 1.0 μg, and 90% mortality after 45 min at 10 μg.

DISCUSSION

All of the 23 phlaeothripine species analyzed contained at least one anal secretion component. A total of 42 components (Table 2) including four that were unidentified (UK-I to UK-IV), were found from 23 species in 17 genera. Of these components, 14 compounds (**11**, **12**, **13**, **14**, **20**, **21**, **27**, **31**, **32**, **33**, **34**, **35**, **36**, and **42**) were detected for the first time. Blum and coworkers reported the components of anal secretions of seven phlaeothripine species in seven genera (Howard et al.,

TABLE 3. REPELLENT ACTIVITY OF CANDIDATE COMPOUNDS AGAINST ANTS, *Formica japonica*

Test compound	Dose (μg)	Total no. of ants ($N = 5$)			
		Control	Treated (%)	Repellency (%)	
Octanoic acid + decanoic acid	0.1	55	45	10	ns
(7: 3 mixture)	1.0	53	47	6	ns
	10.0	55	45	10	ns
β-Myrcene	0.1	50	50	0	ns
	1.0	55	45	10	ns
	10.0	48	52	−4	ns
Citral (mixture of geranial	0.1	42	58	−16	ns
and neral)	1.0	53	47	6	ns
	10.0	82	18	64	**
Perillene	0.1	47	53	−6	ns
	1.0	38	62	−24	ns
	10.0	48	52	−4	ns
3-Butanoyl-4-hydroxy-6-	0.1	53	47	6	ns
methyl-2*H*-pyran-2-one	1.0	55	[a]45	10	ns
	10.0	67	[a]33	34	*

Note. ns: not significant.
[a] Ants were paralyzed.
$^{*}P \leq 0.05$; $^{**}P \leq 0.01$.

1983, 1987; Blum, 1991; Blum et al., 1992). In our studies, terpenes were commonly found in gall-inducing thrips, such as *Liothrips kuwanai*, *Gynaikothrips ficorum*, *Eugynothrips intorquens*, *Thlibothrips isunoki*, and *Varshneyia pasanii*. Of these species, *Thlibothrips isunoki* had only component **30** whereas *Xylaplothrips inquilinus* contained as many as 11 components.

On the other hand, the subfamily Idolothripinae contained only juglone and some carboxylic acids (Suzuki et al., 1990, 2000; Blum, 1991). Thus, the subfamilies can be easily distinguished by the analysis of their secretion components. The genera *Holothrips* and *Apelaunothrips* were first classified into the subfamily Idolothripinae by Priesner (1961), but Mound (1974), Okajima (1979), and Mound and Palmer (1983) transferred them into the subfamily Phlaeothripinae. Our previous results (Suzuki et al., 1993) and present studies of three *Holothrips* species and one *Apelaunothrips* species support the classification of Mound (1974), Okajima (1979) of *Apelaunothrips*, and Mound and Palmer (1983) of *Holothrips* into Phlaeothripinae.

A common inquilinous thrips, *L. piperinus*, can be found in leaf galls of *L. kuwanai*, *G. ficorum*, *V. pasanii*, and *E. intorquens*. The secretion components of this inquilinous thrips are similar to those of the former three species, but not to that of the later species. The larvae of the inquilinous thrips are thought to be predacious on larvae of the gall inducer. β-Acaridial (**29**), a novel monoterpenedial

that was almost simultaneously identified in our laboratory from the acarid mite, *Tyrophagus putrescentiae* (Leal et al., 1989a), and from three thrips species (Suzuki et al., 1989). It was found in all six species belonging to *Liothrips*, *Gynaikothrips*, and *Varshneyia*. One gall inducer, *V. pasanii*, reported previously (Suzuki et al., 1986, 1988, 1989), is not classified in the tribe Hoplothripini, but in the tribe Leeuweniini (Priesner, 1960), but the similarity of its secretion components to those of *Liothrips* and *Gynaikothrips* spp. in the tribe Hoplothripini (in particular, they commonly contained terpene **29**), suggests that its taxonomic placement should be reexamined. The terpene **29** is apt to be overlooked by GC using packed columns. Howard et al. (1987) reported that the secretion of *G. ficorum* was composed of **4** and **16** as major components, together with the minor components tridecane (**2**), tetradecane (**3**), heptadecane (**8**), and tetradecyl acetate (**14**), but they did not refer to **29**. We identified **29** from *G. ficorum* (as mentioned above, *G. uzeli* was reassigned to *G. ficorum*). We had previously overlooked **29** (Suzuki et al., 1986) in GC, using a packed column (SE-30), but detected it using a capillary column (Suzuki et al., 1988, 1989), although it was not eluted from an FFAP capillary column. The four *Liothrips* species analyzed were chemotaxonomically distinguishable by the GC profiles of their secretion components (Figure 1). *Haplothrips aculeatus* and *H. kurdjumovi* secreted only carboxylic acids. However, *H. leucanthemi* emitted only a lactone, mellein (Blum et al., 1992). The acids **21**, **22**, and **23**, and **25** are widely distributed in the acid components of both Phlaeothripinae and Idolothripinae (Blum, 1991; Suzuki et al., 2000).

The function of the components to the ecology of the thrips is thought to be defensive. Citral (a mixture of **31** and **32**) displayed strong repellency against ants at a dose of 10.0 μg, but **28** and **30** displayed no repellency. Compound **41** has an antibiotic activity (Namiki et al., 1952), and was toxic against the ants at doses of more than 1.0 μg. Juglone, plumbagin (**37**), and **25** showed significant repellency in the same tests reported previously (Suzuki et al., 1995). Juglone was most active, showing 66 and 100% repellency at doses of 0.1 and 1.0 μg, respectively. Citral (Okamoto et al., 1981) and terpene **29** (Kuwahara et al., 1989) have antifungal activity, in which the former and the latter have been identified in acarid mites as an alarm (Kuwahara et al., 1980) and a sex pheromone (Leal et al., 1989b), respectively. However, *Liothrips wasabiae*, in which **29** was a major secretion component, is not a gall inducer, although it is gregarious. Further, in *V. pasanii*, GC revealed no difference between males and females in **29** content (Suzuki et al., unpublished data), suggesting that both compounds may protect galls from fungal infestation, as well as playing a defensive role.

It is interesting that several terpenes or aromatic compounds are commonly found from thrips and mites including **28**, **31**, **32**, rosefuran, **29**, **37**, **38**, and **42**. In particular, the latter five compounds have so far been detected in animals only from mites and thrips (for a review on the secretion components of acarid mites, see Kuwahara, 1999). Compound **29** was detected from 21 species of acarid mites,

whereas it was found from six thrips species, but may be widely distributed in the genus *Liothrips*. Compound **42** was found from 15 mite species, and also identified from the thrips, *Xylaplothrips inquilinus* in the present study. Rosefuran was distributed in 13 mite species, such as in the genera *Tyrophagus* and *Caloglyphus*, whereas it was found from the thrips, *Arrhenothrips ramakrishnae* (Blum, 1991). A monoterpene **28** was identified from *L. wasabiae*, *L*. sp., *X. inquilinus*, and *O. frontalis* in the present study, and has also been found from *L. kuwanai*, *L. piperinus*, and *V. pasanii* (Suzuki et al., 1986, 1988, 1989), and *A.ramakrishnae*, *Schedothrips* sp., and *Teuchothrips longus* (Blum, 1991), while it was detected from five mite species (Kuwahara, 1999). Naphthoquinones **37** and **38** have been detected from *Ponticulothrips diospyrosi* and *P*. sp. (Suzuki et al., 1995), whereas they were found only from the mite *Uroactinia hirschmanni* (Sakata et al., 1997). The quinone **37** was further identified from *Dolichothrips* sp. in the present study.

As mentioned above, the subfamily Phlaeothripinae is composed of about 2500 species, and the chemical ecology of thrips is only partly understood (Ananthakrishnan and Gopichandran, 1993). Chemical analysis of anal secretion components is a useful tool for chemotaxonomy, i.e., the classification of tubuliferan species that are difficult to distinguish morphologically. The advantage of using this method is that it is an easier and quicker approach to taxonomic identification than using molecular tools.

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Zapoteca formosa: SULFUR CHEMISTRY AND PHYTOTOXICITY

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Abstract—Chemical analysis of *Zapoteca formosa* extracts show that a variety of volatile sulfur-containing components, including cyclic polysulfides, are responsible for the distinctive odor of its germinating seeds and young plants. The major sulfur-containing compounds include acetyl djenkolic acid, djenkolic acid, taurine, cystine, benzothiazole, dimethyl disulfide, 2,4-dithiapentane, 2-hydroxyethylmethylsulfide, trithiane, 1,2,4-trithiolane, and 1,2,4,6-tetrathiepane. Decreased amounts of djenkolic acid in seedlings and young plants, as compared to the seed, indicate that this is likely the metabolic precursor of the volatile sulfur components. Germination and radicle elongation assays show that germinating seeds of *Zapoteca* and mixtures of volatile sulfur-containing compounds are highly phytotoxic to *Cucumis sativa*, *Lactuca sativa*, *Lycopersicon esculentum*, and *Acacia farnesiana*.

Key Words—*Zapoteca formosa*, Mimosoid legumes, sulfur odor, djenkolic acid, cyclic polysulfides, benzothiazole, dimethyl disulfide, 2,4 dithiapentane, 2-hydroxyethylmethylsulfide, xerophytic, phytotoxicity.

INTRODUCTION

Zapoteca is a small genus of Mimosoid legumes found in Southern Mexico, Central America, and Northern Brazil (Hernandez, 1986). The genus belongs to the tribe Ingeae, a group with origins in wet tropical South America (Irwin, 1981).

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A recurring theme within Ingeae genera is adaptation to more arid environments. For example, *Acacia* has adapted to arid regions of Africa and Australia, and *Mimosa* has diversified in the Planalto of Brazil. *Zapoteca* consists of about 25 species that are highly specialized. They are closely related to the genus *Calliandra*, with which they share a unique fruit structure (Grimes, 1995). *Zapoteca* and *Calliandra* differ in pollen structure (Grimes, 1995), defensive chemistry (Romeo and Morton, 1994), and habitat (Hernandez, 1986). *Zapoteca*, unlike *Calliandra*, is adapted to an arid environment. The differences in chemical defense may be a component in a suite of adaptations that support a xerophytic existence. Field observers (Ataide, Forero, Romeo, unpublished) in the northern state of Bahia in Brazil have noted an unusual patchy distribution of *Zapoteca* species, which suggests that allelopathic interactions might be significant for *Zapoteca* in these habitats.

A distinctive sulfur odor effuses from germinating seeds and roots of *Zapoteca formosa*. This odor, characteristic of *Allium* (leek, garlic, onion), is common to several Mimosoid legumes including *Acacia*, *Albizzia*, *Stryphenodendron*, *Pithecellobium*, *Mimosa*, *Leucaena* (Haines et al., 1987, 1989), and *Parkia* (Mazelis and Fowden, 1973; Gmelin et al., 1981; Susilo and Gmelin, 1982). *S*-alkyl cysteine lyases have been found in high amounts in seeds of *Acacia* and *Albizzia* and act on sulfur containing nonprotein amino acids to produce the odor (Mazelis and Fowden, 1973). The cleavage products derive from compounds such as djenkolic acid, acetyldjenkolic acid, djenkolic acid sulfoxide, dichrostachinic acid, *S*-hydroxy-2-carboxyethylthiomethyl-cysteine, and to a lesser extent *S*-carboxyethyl-cysteine. Identified volatile products include alkyl mercaptans, pyruvate, and ammonia (Mazelis and Fowden, 1973). Piluk et al. (2001) recently reported that the production of carbon disulfide (CS_2) and carbonylsulfide (OCS), which is common in the Mimosoideae, is uncommon in Caesalpinoideae and Papilionoideae legumes. Their studies indicate that CS_2 is produced by the action of cysteine lyase on djenkolic acid (Piluk et al., 1998). They have speculated that CS_2 plays a major ecological role as a deterrent to pathogens (Feng et al., 1998; Piluk et al., 2001).

We undertook a chemical study to identify the volatile odor components of germinating seeds and seedlings of *Z. formosa*. Selected nonvolatile components, including non-protein amino acids, fatty acids, and organic acids were also characterized. Because of field observations suggesting the possibility of allelopathic effects, the identified compounds, as well as *Zapoteca formosa* seeds, were evaluated for phytotoxicity in germination and radicle elongation assays with four test species.

METHODS AND MATERIALS

Biological Material. Seeds of *Z. formosa* were collected by E. Carbono in Northern Columbia. Voucher specimens are on file in the University of South

Florida Herbarium, Tampa, FL. They were germinated in 100 × 15 mm petri dishes on Whatman no. 1 filter paper (Clifton, NJ) or kimwipes soaked in deionized water. Seedlings were transferred to 4-in. pots containing soil, sand, and perlite (3:1:1). Young plants were grown in a GPI growth chamber (GPI Instruments, Winter Haven, FL) under four, 40 W UV cool white lamps (Westinghouse, Philadelphia, PA) under a 14L:10D photoregime at 34 and 32°C, respectively. The average photosynthetically active radiation measured 60 cm from the source was between 180 (sides) and 260 (center) microeinsteins. RH was maintained at 40–50%.

Chemicals. Unless otherwise noted, all solvents and chemicals were reagent grade. Standards of amino acids and of the sulfur-containing compounds benzothioazole, dithiane, trithiane, carbon disulfide, and dimethyl disulfide, were obtained from Sigma Chemical Company (Sigma, St. Louis, MO). A mixture of trithiolane and tetrathiepane was synthesized by a modification of Gil and MacLeod (1981) method. A $Na_2S_{2.5}$ solution was prepared by mixing 100 g $Na_2S_9H_2O$ and 20 g elemental sulfur in 330 ml of distilled water. The mixture was stirred for 13 hr at 26°C. At this time, the solution was a red-orange color. Following filtration, 330 ml of dichloromethane were added, and the solution was vigorously stirred for 8 hr. The organic layer was separated and dried under sodium sulfate. The excess dichloromethane was removed by rotary evaporation leaving a brown oil. This oil was covered with nitrogen and stored at −20°C overnight. Kughlrohr distillation of the oil was performed with a vacuum slightly less than 1 atm. As the brown oil was heated to between 40 and 70°C, yellow oil was collected. The synthate was characterized by ^{13}C and ^{1}H proton NMR (nuclear magnetic resonance, UV (ultra-violet) absorption, and GC–MS.

Amino Acid Extraction. Ground seed and root tissue from *Z. formosa* were extracted (3 times) in chloroform (Morita and Kobayashi, 1966), and extracts were concentrated by rotary evaporation, or by evaporation under a stream of nitrogen. If an aqueous fraction was also needed to analyze less volatile compounds, the initial extraction was in methanol:chloroform:water (12:5:1). Aqueous and organic fractions were separated by adding 1.0 ml of water and 1.5 ml of chloroform to each 6 ml of extract (Singh et al., 1973). Chloroform extracts were stored in 1.5 ml vials at −20°C, or for longer periods at −80°C under reduced pressure in a dessicator. Aqueous fractions were reconstituted in 25% ethanol and stored in Eppendorf vials at 5°C.

Amino Acid Analyses. Methanolic extracts of amino acids were analyzed by two-dimensional paper chromatography (2D-PC; butanol:formic acid:water—15:3:2, followed by 80% aqueous phenol:water in the presence of ammonia vapor); high voltage paper electrophoresis (HVE) (3000 V, pH 1.9 for 30 min) followed by ninhydrin detection; and high performance liquid chromatography (HPLC) on an Aminostat component system analyzer equipped with a 0.5 × 12 cm column packed with DC-5a cation exchange resin (DIONEX MBF-SS amino acid analyzer,

DIONEX Corp., Sunnyvale, CA). Ion-exchange separated the amino acids using a pH gradient of lithium citrate buffers from 2.8 to 6.5 (Lee, 1974). They were detected by OPA (*o*-phthaldialdehyde) fluorescence. The fluorometer of the amino acid analyzer interfaced with a Shimadzu C-R3A chromatopac data processor (Shimadzu, Columbia, MD). Peaks were identified by absolute retention time and quantified by relation to standards of known concentration. The fluorescence detector had wide band excitation and emission filters.

Volatile Collection. Volatile substances emitted from germinating seedlings and roots of mature plants were obtained by modified methods of Tangerman (1986) and Tang and Young (1982), respectively. High purity nitrogen was passed through a closed chamber containing germinating seeds or young seedlings of *Z. formosa*. Volatiles were adsorbed onto a 200 mg Tenax-GC (Alltech, Deerfield, IL) trap, which had been packed into 2 mm ID tubing and cooled by crushed dry ice. A calcium chloride pre-column filter removed water. After absorption, columns were desorbed by heating them to 200°C over 30 min and maintaining this temperature for an additional 20 min. Compounds were captured in small pyrex collection vials cooled with crushed dry ice and containing 1 ml dichloromethane. Samples were stored in sealed vials up to 1 week at −80°C until gas chromatography–mass spectroscopy (GC–MS). All glassware was coated with dimethyldichlorosilane (Sigma Chemical Co.) to minimize surface interactions (Woolley et al., 1986; Jones, 1987).

In other experiments, *Z. formosa* seeds were germinated in a closed 0.5 l glass purge vessel for 5 days. High purity helium (99.999%) was then used to purge the volatiles into a stainless steel trap containing 0.30 g of dry Tenax TA (60–80 mesh). The flow rate was 40 ml/min, and the chamber was purged for 16 hr. Volatiles were desorbed (185°C, 15 min) from the Tenax trap using a Tekmar model 4000 Dynamic Headspace Concentrator (Mason, OH) and flushed through a heated transfer line (120°) into the GC column (60 m × 0.25 mm ID × 0.25 μm film thickness Supelcowax 10 column, Supelco Inc., Bellafonte, PA). During desorption, a 10 cm length of the column at the injector end was held in a liquid nitrogen bath to cryogenically focus the desorbed compounds in the GC column. The carrier gas head pressure was 30 psi, and the septum purge and split vent were closed during desorption. Following desorption, the liquid nitrogen bath was removed and the temperature program begun.

Root Exudate Collection. A modification of the Tang and Young (1982) system was used to trap root exudates from roots of mature plants that we did not want to sacrifice. XAD-4 resin (20–50 mesh, Alltech # 5781, Deerfield, IL) was packed into columns (14 cm long × 15 mm ID; Pyrex, Corning, Danville, VA). Plants were removed from pots and placed into funnels filled with aquarium and basalt rock (autoclaved) that connected to the columns. They were watered with sterile Hoagland's solution for 3 days (Epstein, 1972). Solutions recirculated through the XAD columns during this time. After 72 hr, columns were detached and

washed with five successive bed volumes of sterile distilled water, methanol, and dichloromethane to elute the root exudate. Fractions were concentrated by rotary evaporation. The water and methanol fractions were combined and reconstituted in 25% ethanol and stored in a refrigerator. The dichloromethane fraction was dried over sodium sulfate, concentrated, and stored under nitrogen at -80°C.

Mass Spectral Analysis. GC–MS was performed on an Hewlett Packard (HP 5992B) system equipped with a quadrupole electron impact detector at 70 eV (electron volts), or an HP 5792 gas chromatograph with model 5970 mass selective detector (Hewlett Packard, Avondale, PA). On the HP 5992B, the column was a 30 m $\times$ 0.25 mm ID DB-5 fused capillary column. Samples were injected into an oven at 24°C, the temperature was increased at a rate of 2°C/min for 5 min. This was followed by 32 min at 280°C, and then a maximum temperature of 300°C for 1 min. The computer integrator (9825B) had an attached dual disc drive (9885M/S) for computer matching mass spectral data with EPA/NIH library files. Extract spectral data were also compared with standards and control runs for each experiment, as well as published spectral data for cyclic polysulfides (Morita and Kobayashi, 1966, 1967; Gil and MacLeod, 1981; McLafferty and Stauffer, 1988).

Thin Layer and High Pressure Liquid Chromatography Analyses. TLC was performed on silica gel G plates (Kodak#13181) in several systems (Kodak, Rochester, NY). Solvents used were chloroform; chloroform:benzene (2:3 (v/v)); toluene:ethyl acetate (9:1); hexane:ethyl acetate (varying ratios); and cyclohexane. Spots were detected under 366 nm ultraviolet, by iodine fuming, or iodoplatinate and dichlorodicyanobenzoquinone.

HPLC was performed on chloroform extracts on an Altex (model 153) system (Danberry, CT) equipped with a 254 nm UV detector, integrated with a Shimadzu CR-3A chromatopac data processor (Shimadzu, Columbia, MD), and a reversed phase column (C-18, 25 cm $\times$ 5 mm ID; Waters, Milford, MA). A methanol (HPLC grade):water (4:1) mobile phase flowed at 0.5 ml min^{-1} at 1200 psi. Peaks were identified by absolute retention time. Detection limits of the HPLC system were 5×10^{-6} g ml^{-1} for dithiane (4.2×10^{-7} moles), 7.6×10^{-7} g ml^{-1} for carbon disulfide (1×10^{-8} moles), and 9.4×10^{-4} g ml^{-1} for dimethyl disulfide (9.4×10^{-4} moles). Experimental data were compared with HPLC separation of sulfur compounds in the literature (Chen and Ho, 1986).

Fatty Acids (FA) and Organic Acids (OA). Derivatized compounds were analyzed by gas chromatography [Sigma 2000 Perkin-Elmer (Boston, MA)]. The GC was equipped with a LCI-100 integrator and a flame ionization detector, with helium used as the carrier gas, or by GC–MS.

Bioassays. For tests of phytotoxicity, two different bioassays were used: 1) Test species were paired with germinating *Z. formosa* seeds on Whatman no. 1 filter paper (Clifton, NJ) in petri dishes, or test species were placed on papers moistened with test-compounds, or 2) seeds were placed onto 3 cm high platforms so they did not come into direct contact with seeds or test compounds on

moistened papers located below. They were, thus, exposed only to emitted or test volatiles. Density–volume relations were taken into consideration (Weidenhamer et al., 1987). Test species included *Cucumis sativa, Lactuca sativa, Lycopersicon esculentum,* and *Acacia farnesiana*. The number of seeds used per dish for germination and radicle elongation assays were *Cucumis sativa* (5 seeds), *Acacia farnesiana* and *Lycopersicon esculentum* (25 seeds), and *Lactuca sativa* (50 seeds). Germination rates were 100% for *L. sativa* and *L. esculentum*, 85% for *A. farnesiana,* and 92, 94, and 98% for *C. sativa* at 24, 48, and 72 hr, respectively. All bioassays used 2 ml of solution unless otherwise noted. Six control replicates and 10 experimental replicates were performed. In the time-course study, water-soluble leachates were removed from seeds and seedlings on a daily basis and used as test solutions. Controls, containing only distilled water, had the same number of seeds as experimental treatments.

RESULTS AND DISCUSSION

Chemistry: Seeds and Root Extracts. Sulfur compounds detected in seeds of *Z. formosa* include the nonprotein amino acids djenkolic acid and acetyldjenkolic acids, the amine taurine, and benzothiazole (Table 1). Cystine, an oxidation product of two cysteine amino acids, was found but only in root exudates. Djenkolic acid is found in other Mimosoids including *Acacia, Albizzia, Pithecellobium,* and *Dichrostachys* (Krauss and Reinbothe, 1970), *Desmanthus, Leucaena, Mimosa, Neptunia,* and *Prosopis* (Piluk et al., 2001). Additionally, seed extracts contained several common fatty acids (including hexanedioic, heptanedioic, octanedioic, nonanedioic acids), organic acids (including lactic, malonic, succinic acids), and 10 common benzene derivatives (including napthalenes, phthalates, and *N*-benzylglycine) and the less common 1,1′-(1,2-ethanediyl)bis-benzene, known from Australian liverworts (Asakawa et al., 1981) and a *Cymbidium* orchid (Juneja and Tandon, 1987).

Root extracts contained a number of non-sulfur volatiles, including 1,1-diethoxyethane, 2-octanol, and *N*-benzylglycine. Organic and fatty acids decreased significantly from their greater concentrations in seeds (data not shown).

Chemistry: Root Exudate. The strong, garlic-like odor that appeared within 2 days of adding water to the *Z. formosa* seeds was persistent and continued to emanate from the growing roots for up to 3 months in laboratory grown plants. Components of this odor (Table 1, Figure 1) from 5-day old seedlings include benzothiazole, dimethyl disulfide, 2,4-dithiapentane, 2-hydroxyethylmethylsulfide, and three rare cyclic polysulfides, 1,2,4,6-tetrathiepane, and 1,2,4-trithiolane, both known from *Parkia* (Gmelin et al., 1981), and 1,3,5-trithiane known from Shiitake mushrooms (Chen and Ho, 1986). Carbon disulfide, recently identified from six other Mimosoids (Piluk et al., 2001), was not detected, however, its derivative

TABLE 1. SULFUR-CONTAINING AND OTHER COMPOUNDS IDENTIFIED IN *Zapoteca formosa* SEEDS, GERMINATING SEEDS, AND ROOT EXUDATES

Chemical compound	Identification technique	*Zapoteca formosa* tissue
Acetyl djenkolic acid	HVE, 2D-PC, HPLC	Seeds
Djenkolic acid	HVE, 2D-PC, HPLC	Seeds[a]
	HPLC	Germinating seeds
		Root exudates
Cystine	HVE, 2D-PC, HPLC	Root exudates
Taurine	HVE, 2D-PC, HPLC	Seeds
		Germinating seeds
		Root exudates
Benzothiazole	GC/MS	Seeds
		Germinating seeds
		Root exudates
1,1-Diethoxyethane	GC/MS	Germinating seeds
Dimethyl disulfide	GC/MS	Germinating seeds
2,4-Dithiapentane	GC/MS	Germinating seeds
2-Hydroxyethylmethylsulfide	GC/MS	Germinating seeds
2-Octanol	GC/MS	Germinating seeds
1,3,5-Trithiane	TLC	Germinating seeds
		Root exudates
1,2,4-Trithiolane	TLC, HPLC	Germinating seeds
		Root exudates
1,2,4,6-Tetrathiepane	TLC, HPLC	Germinating seeds
		Root exudates
Organic & fatty acids	GC	Seeds
		Germinating seeds
		Root exudates
Benzene derivatives	GC/MS	Seeds

[a] Concentrations of djenkolic acid in seeds were in the range >100 to 200 nmole per seed range, whereas in germinating seeds and root exudates they were <100 nmole per plant.
GC = gas chromatography; GC/MS = gas chromatography/mass spectroscopy; HPLC = high performance liquid chromatography; HVE = high voltage electrophoresis; TLC = thin layer chromatography; 2D-PC = two-dimensional paper chromatography.

dimethyl disulfide was found. It is likely that carbon disulfide is a component of the *Z. formosa* odor, however, our system was not optimized for its detection. Cyclic polysulfides have a sporadic occurrence throughout the plant and fungal kingdoms. Besides Mimosoids and Shiitake mushrooms, they have been reported from other Basidiomycestes (Gmelin et al., 1980), a red alga, *Chondria californica* (Wratten and Faulkner, 1976), a green alga, *Chara globularis* (Anthoni et al., 1980), hops (Elvidge and Jones, 1982), wood garlic (Oleaceae) (Lim et al., 1998), and *Allium*, onion (Freeman and Whenham, 1975; Kameoka and Demizu, 1979) and garlic (Freeman and Whenham, 1975; Block et al., 1986).

Gmelin et al. (1957) found an enzyme in *Albizzia lophantha*, cysteine sulfoxide lyase, that acts on djenkolic acid and its acetyl derivative to produce

methanedithiol. Methanedithiol was later found to be an unstable intermediate in the formation of cyclic polysufides in *Parkia speciosa* (Gmelin et al., 1981). Piluk et al. (2001) found that both djenkolic acid and cysteine lyase were required for CS_2 production. Although we did not perform enzyme studies, concentrations of djenkolic decreased by approximately half (data not shown) in the root exudates from 3 month old plants as compared with the amount found in seed extracts.

Sulfur odors in plants are known to be complex. In *Allium*, they arise from mixtures of thiosulfinates, aliphatic sulfides, and cyclic polysulfides (Block et al., 1986; Vernin et al., 1986). Cysteine derivatives are broken down by an enzyme, allinase, into various thiosulfinate products (Stoll and Seebeck, 1951). These are further reduced to several aliphatic and cyclic polysulfides (Freeman and Whenham, 1975; Block et al., 1986). In *Scorodocarpus borneensis*, the odor is a mixture of di- and polysulfides (Kubota et al., 1998; Lim et al., 1998). The sulfurous aroma of shiitake mushrooms, is a mixture of 18 cyclic and noncyclic sulfur compounds including several cyclic polysulfides, carbon disulfide, dimethyl disulfide, carbon trisulfide, and carbon tetrasulfide (Chen and Ho, 1986). In the case of *Z. formosa*, the odor is due to sulfur-containing compounds of a variety of structural types (Table 1, Figure 1). Furthermore, the biological activity (see below) is clearly attributable to a mixture rather than a single compound.

Bioassays. Germinating seeds of *Z. formosa* are highly phytotoxic (Table 2). Common bioassay species were all strongly affected. A single *Z. formosa* seed, when watered and placed amidst 50 *L. sativa* seeds, 25 *L. esculentum* seeds, or 25 *A. farnesiana* seeds, another species that contains cyclic polysulfides, completely inhibited all germination in petri dish assays. *C. sativa* was less sensitive. When five *C. sativa* seeds were paired in the same way with three seeds of *Z. formosa*, germination was 33% at 24 hr, 53% at 48 hr, and 54% at 72 hr (Table 2). Aqueous leachates of *Z. formosa* reduced *C. sativa* germination to 83% (Table 3), however, volatiles had no effect (data not shown).

It is unclear what chemicals are responsible for the phytotoxic effects. Surprisingly, tests of individual compounds at 0.5 mmole (including benzothiazole, djenkolic acid and 2-octanol) showed no or little effect. Benzothiazole, djenkolic acid, and 2-octanol had no effect on germination of *C. sativa*, and only 2-octanol had any effect on *C. sativa* radicle elongation, reducing radicle elongation by 36% (data not shown). A 0.5 mmole 1:1 mixture of cyclic polysulfides, 1,2,4-trithiolane, and 1,2,4,6-tetrathiepane, however, was inhibitory to seed germination of three species and radicle elongation of all four test species (Table 3). Other combinations of these and other *Z. formosa* compounds need to be tested to pinpoint the source of the activity, and tests with native species that occupy *Z. formosa* habitats, of course, are needed.

Inhibitory growth effects on emerging *C. sativa* radicles were more dramatic. In time-course experiments in which water leachates of germinating *Z. formosa* were tested daily, the growth effect was 10.4% of controls after 24 hr (Table 3).

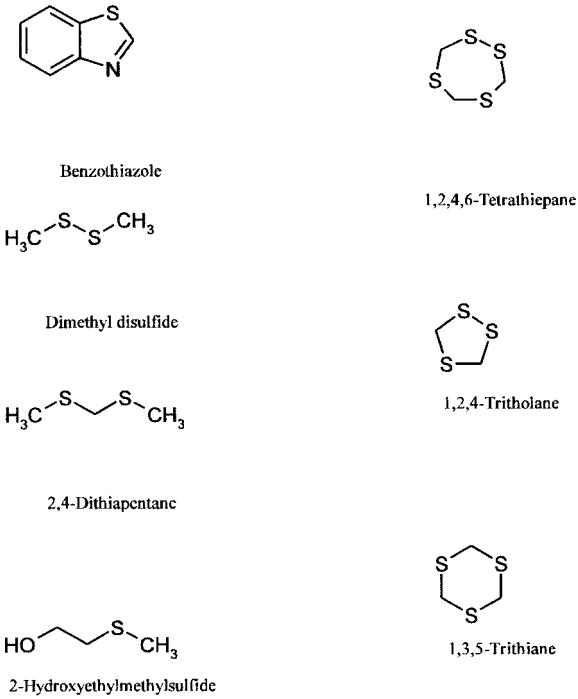

FIG. 1. Components of the sulfurous odor emitted from *Zapoteca formosa*.

This was maintained with exudates up to day 3, decreasing slightly each day, to day 8 when the experiment was terminated.

Ecological and Evolutionary Implications. Z. formosa plants in the state of Bahia in Northern Brazil are surrounded by bare zones that are regularly and

TABLE 2. EFFECT OF CO-GERMINATING *Zapoteca formosa* SEEDS ON THE GERMINATION OF TEST SPECIES

Test species	Germination (% of control)[a]
Cucumis sativa	
24 hr	33
48 hr	53
72 hr	54
Acacia farnesiana	0
Lactuca sativa	0
Lycopersicon esculentum	0

[a] *Cucumis sativa* seeds were co-germinated with three *Z. formosa* seeds. Other species were co-germinated with one *Z. formosa* seed.

TABLE 3. EFFECT OF *Zapoteca formosa* EXTRACTS AND A 1:1 MIXTURE OF TRITHIOLANE AND TETRATHIEPANE ON GERMINATION AND RADICLE ELONGATION OF TEST SPECIES

Test species	Treatment	Germination (% of control)	Radicle growth ± SE (mm)
Cucumis sativa			
24 hr	*Z. formosa* aq. extract		10.4 ± 3.4
48 hr			11.0 ± 6.4
72 hr		76	13.3 ± 10.2
Cucumis sativa	Trithiolane/tetrathiepane	No effect	66.8 ± 11.6
Acacia farnesiana	Trithiolane/tetrathiepane	3.0	[a]
Lactuca sativa	Trithiolane/tetrathiepane	47	15.4 ± 3.4
Lycopersicon esculentum	Trithiolane/tetrathiepane	82	30.1 ± 7.2

[a] Because of the fact that only three seeds germinated, radicle growth data are not presented.

strikingly spaced in a way similar to allelopathic plants of the American west and Florida scrub communities. This observation coupled with the high phytotoxicity observed in these studies, suggests that the allelopathic potential of this plant should be investigated. Sulfur-containing compounds have a wide array of biological activities. Heterocyclic sulfur compounds are known urease inhibitors (Gould et al., 1978), cyclic polysulfides can inhibit photosynthesis (Anthoni et al., 1980), and disulfides are potent inhibitors of nitrification (summarized in Piluk et al., 2001). Potential roles of such compounds for young seedlings could include protection from pathogens and nematodes, as well as inhibition of neighboring seedlings. Further experiments are warranted to determine the ecological function(s) of these compounds for *Z. formosa*.

There is essentially universal taxonomic agreement that the tribe Ingeae is the more advanced among the Mimosoids (Elias, 1981), and that Mimosoids in general appear to have their origins in wet tropical South America (Irwin, 1981). *Zapoteca*, however, occupies sandy soils in harsh, xeric habitats. We suggest that the sulfur chemistry of *Z. formosa* may play a role in its adaptations to a xeric habitat. Its seeds contain the rare sulfur amino acids, djenkolic and acetyldjenkolic acids (Romeo, 1986). These amino acids, metabolized quickly upon germination, lead to cyclic phytotoxic compounds reported here. *Zapoteca*, with its restricted distribution in arid habitats, contrasts sharply with *Calliandra*, its nearest relative. *Calliandra* is found typically along riverbanks and on rocky outcrops in the wet tropics. Its seeds and young leaves are well fortified against herbivores with an array of imino and sulfur amino acids (*S*-β-carboxyethylcysteine and *S*-β-carboxyisopropylcysteine) (Romeo and Simmonds, 1989), but its roots produce no garlic-like odor, nor do they exude any sulfur compounds. We note, however, that several other rain forest legumes do produce sulfurous emissions (carbon disulfide) from their roots (Haines et al., 1987, 1989). In the wet tropics, protection against seed and leaf herbivory

would be expected to be selectively favored, as those pressures are intense. In contrast, in the drier habitats favored by *Zapoteca*, plant competition for nutrient and water resources may be the decisive factor in survival.

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Casuarina cunninghamiana TISSUE EXTRACTS STIMULATE THE GROWTH OF *Frankia* AND DIFFERENTIALLY ALTER THE GROWTH OF OTHER SOIL MICROORGANISMS

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Abstract—Aqueous extracts of host plant *Casuarina cunninghamiana* tissue altered the *in vitro* growth of its diazotrophic microsymbiont *Frankia* and a selection of other soil microorganisms. The growth of actinomycetous *Frankia* strains, 55005, AvcI1, CesI5, CjI82 001, and Cj was stimulated by aqueous extracts of *C. cunninghamiana* tissue. Green cladodes (photosynthetic branches), unsuberized roots, and suberized roots were more stimulatory than dry cladodes and seed tissue. Aqueous extracts of green cladodes of *C. cunninghamiana* most stimulated the growth of *Casuarina*-derived *Frankia* strains CjI82 001 and 55005. The growth of isolates of soil bacteria *Bradyrhizobium japonicum*, *Arthrobacter globiformis*, and *Bacillus subtillis* and of the soil fungi *Penicillium oxalicum* and *Arthroderma cookiellum* was either inhibited or not affected by cladode extracts. Cladode extracts stimulated the growth of the actinomycete *Streptomyces albus* and the fungus *Rhizopus homothallicus*. The magnitude (as great as 100%) of the increase in growth caused by tissue extracts for the *Casuarina*-derived *Frankia* strains relative to other soil microbes suggests a host-specific enhancement of the microsymbiont.

Key Words—*Frankia, Casuarina, Terminalia,* actinorhizal plants, symbiosis, soil microbial community, rhizosphere.

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INTRODUCTION

Frankia, a genus of actinomycetes, forms root nodules with 25 genera of actinorhizal plants (Baker and Schwintzer, 1990) including *Casuarina,* resulting in symbiotic nitrogen fixation. *Frankia* infectivity in soil is typically greater near hosts (Wollum et al., 1968; Zimpfer et al., 1999) and a few related nonactinorhizal plants (Smolander, 1990; Paschke and Dawson, 1992; Gauthier et al., 2000). The reasons for this localization are not known, though *Frankia* can produce spores within nodules that could be released allowing an infective form to persist in soil in increased numbers (Tisa et al., 1983).

Actinohizal plants may release compounds that benefit symbiotic function (Kapulnik et al., 1987). Benoit and Berry (1997) determined that compounds present in actinorhizal *Alnus* host tissue may be directly involved in host/symbiont signaling and infection processes. *Alnus* and *Elaeagnus* root extracts increase *Frankia* spore germination (Krumholz et al., 2003). There are numerous reports of host plants favoring specific members of the soil microbial community. *Frankia* proliferates in the rhizospheres of *Alnus glutinosa* (Vergnaud et al., 1985), *Betula* (Smolander, 1990; Paschke and Dawson, 1992), and *Alphitonia* (Gauthier et al., 2000). None is actinorhizal, but they are members of plant families with actinorhizal genera. *Frankia* is localized in soil near *Casuarina cunninghamiana* (Zimpfer et al., 1999). Maunuksela et al. (1999) found different populations to exist in alder capture plants depending on whether the soil originated from birch, pine, or spruce stands. Nickel (2000) found different subgroups of alder-infective *Frankia* in nodules of capture plants dependent upon on whether the soil was amended with dried alder leaves. These findings suggest that hosts may favor *Frankia* in their rhizospheres.

Plants can also selectively promote root bacteria (Wilkinson et al., 1994; Lemanceau et al., 1995; Latour et al. 1996, 1999; Sampo et al., 1997; Elo et al. 2000) and mycorrhizal fungi (Dickie et al., 2002) from soil. They influence the growth of microorganisms and affect soil community structure through secondary compounds (Whitehead, 1964; Li et al., 1970; Harborne, 1973; Whitehead et al., 1983; Selim et al., 1996; Sayed and Wheeler, 1999). Selim et al. (1996) demonstrated that growth of *Frankia* isolates from *Casuarina* could be promoted with various fatty acids when grown in pure culture.

Zimpfer et al. (1999) found that detectable levels of *Casuarina*-infective *Frankia* were restricted to within 20 m of *Casuarina* hosts in Jamaica. Additionally, the infectivity of both naturally occurring (Zimpfer et al., 2002) and an isolate of *Frankia* (Zimpfer et al., 1999) were increased by the addition of aqueous extracts of *Casuarina* cladodes.

The ability of *Casuarina* cladode tissue to alter the infectious capacity of *Casuarina*-infective *Frankia* (Zimpfer et al., 1999) led us to hypothesize that compounds present in *Casuarina* tissue are able to stimulate the growth of *Frankia* and inhibit the growth of other soil microorganisms.

METHODS AND MATERIALS

Influences of Aqueous Extracts of C. cunninghamiana *Tissues on* Frankia *Growth.* *Frankia* strains (Table 1) were grown in 20 ml of a liquid propionate-BAP medium (Murry et al., 1984) in sterile 50 ml centrifuge tubes. The culture medium was amended with aqueous extracts of different tissue types of *C. cunninghamiana* added to make media containing extracts from 5 g of fresh tissue per l. This concentration had previously been shown to affect the infectious capacity of an artificial soil inoculated with *Frankia* (Zimpfer et al., 1999). Extracts were prepared by adding 50 g of green cladodes, senesced cladodes, suberized roots (1–3 mm diam), unsuberized roots, or seeds to 1 l of deionized distilled water (DDW) and homogenizing thoroughly in a blender. Extracts were filtered through filter paper (Whatman No. 4, Springfield Mill, England) and filter sterilized by passing through a 0.2 μm syringe filter. There were four replicate tubes of each type of tissue extract inoculated for each combination of *Frankia* (55005, AvcI1, Cesi5, CjI82 001, or Cj) and 1-ml aliquots containing 660, 465, 400, 340, or 200 μg of *Frankia* protein. Prior to inoculation, *Frankia* colonies were homogenized using a sterile glass tissue grinder. After inoculation, tubes were shaken vigorously once per day. Four days after inoculation, the contents of the tubes were washed (using centrifugation at 2,000 rpm ($650 \times g$) in a clinical centrifuge for 15 min) three times with DDW, and the protein content of the tubes was determined according to the methods of Bradford (1976) with high purity Bovine Albumin as a standard. For each strain, a one-way analysis of variance was performed on protein concentration per ml in a culture tube to determine if there were differences in growth due to the addition of aqueous extracts of tissue types. When the overall effects of tissue extracts were statistically significant, differences between tissue type means were determined using least significant differences (LSD).

Effects of Photosynthetic Tissue on Growth of Isolates of Soil Microorganisms. Common soil bacteria and fungi were chosen. Bacteria were grown in liquid media,

TABLE 1. *Frankia* STRAINS AND SOURCES OF ISOLATES

Strain	Strain no.	Isolated from	Reference or source
55005	DDB 02060510	*Casuarina equisetifolia* (atypical, nonhost infective)	Baker, 1987
AvcI1	DDB 01020110	*Alnus viridis* ssp. *Crispa* (Dryand. Ex Ait.) Turrill	Baker and Torrey, 1980
CeSI5	UFG 026605	*Casuarina equisetifolia*, subcultured from a single spore (atypical, nonhost infective)	Tzean and Torrey, 1989
CjI82 001	ORS 021001	*Casuarina junghuhniana*	Diem et al., 1983
Cj	DDB 000320	Soil beneath *Ceanothus jepsonii* Greene	D. D. Baker

and fungi were grown on agar media appropriate for each species as detailed below. Aqueous extracts of *C. cunninghamiana* cladodes were prepared by homogenizing fresh green tissue in a blender, followed by filtering through cheese cloth. Two hundred and fifty g. of tissue suspended in 1 l of DDW were then passed through filter paper and filter sterilized by passing through a 0.2-μm syringe filter. Aqueous extracts of leaves of *Terminalia catappa*, an exotic tree in Jamaica, where it occurs sympatrically with *C. cunninghamiana*, were added to a subset of cultures of the same soil microorganisms to determine whether the effects were associated generally with plants. Aqueous extracts of *T. catappa* leaves were prepared in the same manner as *C. cunninghamiana* cladodes.

Aqueous extracts of *C. cunninghamiana* cladodes were added to cultures of *Casuarina*-infective *Frankia* strains CjI82 001 and 55005 grown in the medium of Burggraaf and Shipton (1982) with propionic acid as the carbon source. Aqueous extracts of *C. cunninghamiana* cladodes were also added to cultures of *Bradyrhizobium japonicum*, *Arthrobacter globiformis*, *Streptomyces albus*, and *Bacillus subtillis*. Extracts from 0, 2, 5, or 10 g fresh cladode tissue were added per l of medium. Strain numbers and description of the growth media for these species are in Table 2. Tubes containing 10 ml of the appropriate medium were inoculated with 1 ml of bacteria in log growth phase. *Frankia* cultures were incubated at 30°C and shaken at 150 rpm. The remaining bacterial cultures were incubated at 24°C and shaken at 250 rpm. For each species, we determined if differences in tissue concentration in the media affected microbial growth from the time of inoculation through the growth phase. Rates of microbial growth for all the strains of bacteria were estimated by the amount of cellular protein accumulated with growth in the

TABLE 2. SPECIES, PHYLUM/CLASS, STRAIN NUMBER AND GROWTH MEDIA OF NON-*Frankia* BACTERIA AND FUNGI

Species		Strain number	Culture media
Bacteria	Phylum		
Bradyrhizobium japonicum	Proteobacteria	USDA 110	Somasegaran and Hoben, 1985
Arthrobacter globiformis	Firmicutes	ATCC 8010	Difco 0003[a]
Streptomyces albus	Actinobacteria	ATCC 3004	Difco 0370 plus 10% sucrose, w/v
Bacillus subtillis	Endospora	ATCC 23059	ATCC 273[b]
Fungi	Class		
Rhizopus homothallicus	Zygomycetes	ATCC 42221	ATCC 336
Penicillium oxalicum	Deuteromycetes	ATCC 64198	ATCC 336
Arthroderma cookiellum	Ascomycetes	ATCC 56031	ATCC 28

[a] Difco, Sparks, MD, USA.
[b] ATCC (American Type Culture Collection) Manassas, VA, USA.

culture tubes. After two cycles of centrifuging at 2000 rpm for 15 min in a clinical centrifuge and washing twice with DDW, cellular protein was determined by using a modified Bradford method according to Sedmak and Grossberg (1977), with high purity bovine serum albumin as a standard.

The effects of aqueous extracts of *C. cunninghamiana* cladodes on fungal growth were determined for *Rhizopus homothallicus*, *Penicillium oxalicum,* and *Arthroderma cookiellum.* Strain numbers and descriptions of the growth media are listed in Table 2. Filter sterilized extracts from 0, 2, 5, or 10 g of fresh *C. cunninghamiana* cladodes were incorporated into 1 l of medium appropriate for each species. Fungal strains were grown in 100 × 15 mm petri dishes containing 20 ml of an agar medium. Extracts were added after the agar had cooled to 50°C. A 5-mm circular plug extracted from an actively growing culture was placed upside-down on the agar-filled petri dish. Four petri dishes were prepared for each combination of fungal species and level of extract. To determine whether the observed growth stimulation of *R. homothallicus* by extracts was specific to *Casuarina,* aqueous extracts of leaves *Terminalia catappa* were added to petri dishes of media before inoculation. The dishes were incubated in a growth chamber at 24°C. Fungal growth was measured by determining the diameter transecting the center of the colony along three axes of 45°, 90°, and 135°. *Arthroderma cookiellum* cultures were measured along four additional axes 60°, 75°, 105°, and 120° from the center point of inoculation, due to the irregularly shaped colonies. The area of each colony was estimated as π (1/2 mean diameter)^2.

For bacteria in liquid culture, one-way analyses of variance were performed for each organism and for each time of sampling to determine whether there were differences in growth associated with extract concentration. Differences between individual means within species were determined using least significant differences (LSD) for each time of sampling. For fungal samples, repeated measures analyses of variance were performed to determine the significance of differences in growth over time. The estimated area of the fungal colony was the dependent variable for each combination of strain, photosynthetic tissue type, and concentration.

RESULTS

The addition of tissue extracts had a significant ($P < 0.05$) effect on growth (cellular protein accumulation) of *Frankia* strains 55005, AvcI1, CesI5, CjI82 001, and Cj in liquid media (Table 3). Aqueous extracts of green cladodes, fine roots, or seed tissue resulted in greater growth of *Frankia* strain 55005 (Figure 1, $P < 0.01$). Strain AvcI1 had a growth response to green cladodes, fine roots, suberized roots, or seed tissue (Figure 1, $P < 0.01$). For strain CesI5, only extracts of suberized root tissue resulted in more growth than control cultures (Figure 1, $P < 0.01$). Strain CjI82 001 responded to green cladodes, suberized roots, senesced

TABLE 3. SUMMARY OF ONE-WAY ANOVA'S FOR EFFECTS OF AQUEOUS EXTRACTS OF *Casuarina cunninghamiana* TISSUE ON GROWTH OF *Frankia* IN LIQUID MEDIA

Strain	df	Mean square for variation due to extract treatment	Mean square error	*F* value
55005	5	50.15	3.88	12.93**
AvcI1	5	26.91	1.39	19.44**
Cesi5	5	70.45	25.17	2.80*
CjI82 001	5	121.45	3.047	39.86**
Cj	5	22.57	6.70	3.37*

* $P < 0.05$; ** $P < 0.01$.

cladodes, or seed tissue with more growth than control cultures (Figure 1, $P < 0.01$). For strain Cj, extracts of green cladodes, fine roots, suberized roots, or seed tissue resulted in greater growth (Figure 1, $P < 0.05$).

Extracts of fresh *C. cunninghamiana* cladodes (5 g per l) in liquid cultures of *Frankia* strain CjI82 001 resulted in growth stimulation at every sample time ($P < 0.05$). Two or 10 g of fresh *C. cunninghamiana* cladodes per l stimulated growth to a lesser degree. Cultures of CjI82 001 with extracts from 5 g of fresh *C. cunninghamiana* cladodes resulted in more than a fivefold increase in growth compared with the controls after three days (Table 4). Adding leaf extracts of *T. catappa* to *Frankia* strain CjI82 001 cultures at the same concentrations as *C. cunninghamiana* cladodes inhibited growth (Table 4). Cladode extracts ($P < 0.05$) increasd the growth of *Frankia* strain 55005, with extracts from 10 g of fresh *C. cunninghamiana* cladodes per l being the most stimulatory (Table 4).

The growth of *S. albus* cultures was increased ($P < 0.05$) by extracts from *C. cunninghamiana* cladode tissue (Table 5). All concentrations had stimulatory effects during the active growth phase of the cultured organisms. The growth of *B. japonicum* was ($P < 0.05$) decreased by *C. cunninghamiana* cladode tissue (Table 5). All concentrations inhibited growth until the last time of sampling when growth in culture had slowed. At most sampling times, the growth of *B. subtillis* cultures was not significantly affected by the addition of *C. cunninghamiana* cladode tissue extracts; however, there was a general trend of decreasing growth with increasing tissue concentration (Table 5). After 2 and 8 hr the higher concentrations of tissue decreased the growth of *A. globiformis* cultures ($P < 0.05$).

The rate of growth of the fungus *R. homothallicus* was increased ($P < 0.01$) by aqueous extracts of *C. cunninghamiana* cladodes (Figure 2a), however, extracts of leaf tissue of *T. catappa* did not stimulate growth (Figure 2d). The rate of growth of *P. oxalicum* was not affected by aqueous extracts of *C. cunninghamiana* cladode (Figure 2c), while the rate of growth of *A. cookiellum* was inhibited ($P < 0.01$) (Figure 2b).

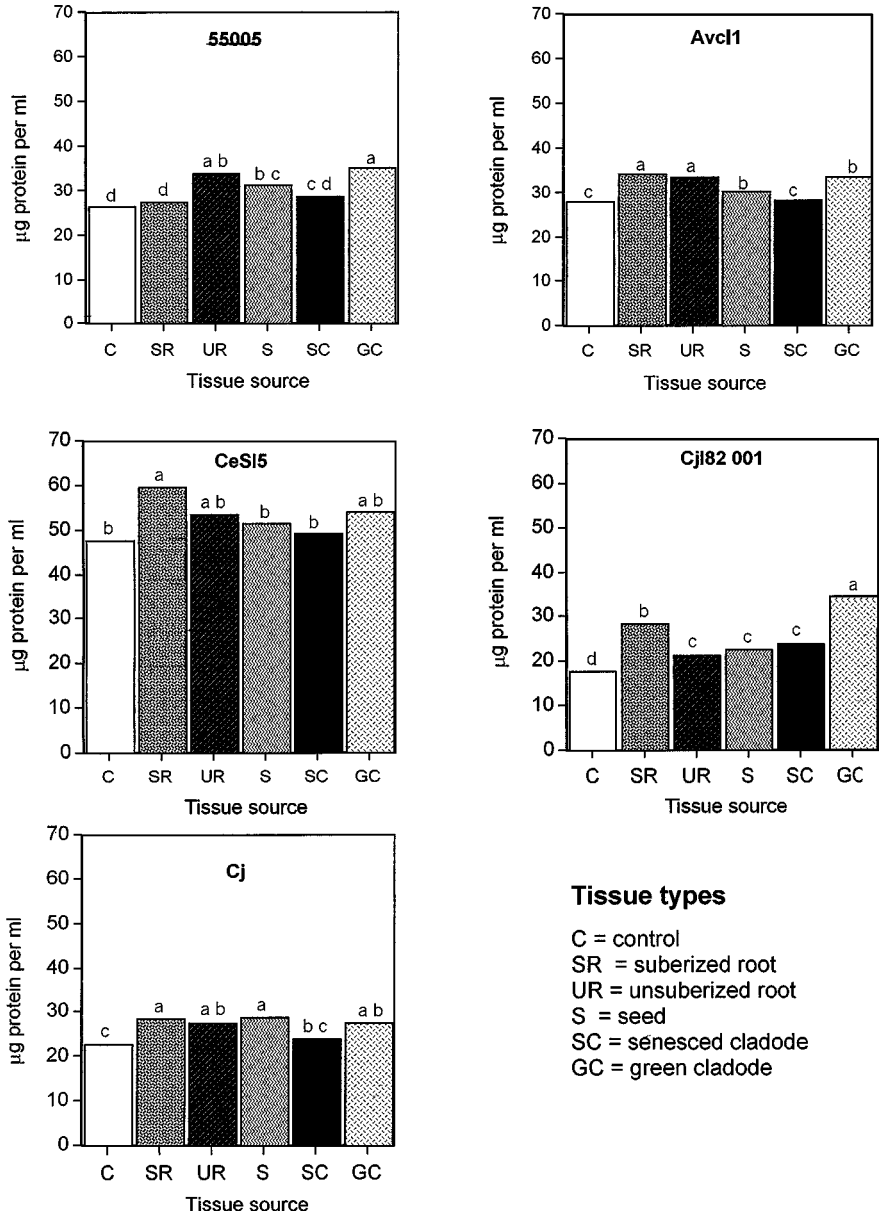

FIG. 1. Effects of aqueous extracts of *Casuarina cunninghamiana* tissue (5 g fresh weight per l of medium) on growth of *Frankia* strains 55005, AvcI1, CeSI5, CjI82 001, and Cj grown for 4 d in 20 ml of liquid medium. Bars within strain with same letter are not significantly different ($P = 0.05$).

TABLE 4. MEAN GROWTH (PROTEIN ACCUMULATION)[a] OF *Frankia* STRAINS CJI82 001 AND 55005 WITH AQUEOUS EXTRACTS OF *Casuarina cunninghamiana* CLADODES AND *Terminalia catappa* LEAVES ADDED TO THE MEDIUM

Frankia strain	Days of growth	Cladode concentration (g/l)			
		0	2	5	10
55005		*Casuarina cunninghamiana*			
	0	0.5	0.5	0.5	0.5
	5	12.9	20.5	20	22
	7	16.1b[b]	21.7ab	23.7a	26.5a
	10	19.8b	23.5b	24.5ba	30.4a
	14	25	27.9	33.7	32.4
	21	22.2b	36.7a	36.9a	36.8a
CjI82 001		*Casuarina cunninghamiana*			
	0	0.2	0.2	0.2	0.2
	3	2.8c	13.6a	12.6a	5.1b
	5	7.0b	9.1b	15.0a	16.7a
	7	12.8b	16.6ab	24.3a	17.6ab
	14	21.8b	30.6a	38.3a	20.2b
	21	23.d	29.c	41.1a	34.4b
CjI82 001		*Terminalia catappa*			
	0	1.5	1.5	1.5	1.5
	3	13.9a	4.1b	10.0ab	3.7b
	4	22.2a	17.7a	17.3a	6.1b
	5	27.6a	17.4ab	15.0b	11.7b
	14	28.6	20.4	21.1	18.4
	21	36.1a	24.1b	29.8ab	20.7b

Note. Frankia were grown in glass tubes containing 10 ml of liquid medium ($N = 3$).
[a] Microgram protein ml^{-1} medium.
[b] Where there were a significant variation in response to treatments in ANOVA, means in a row for each *Frankia* strain and tissue type followed by the same letter are not significantly different ($P < 0.05$).

DISCUSSION

Extracts of *C. cunninghamiana* tissue significantly increased the growth of *Frankia* isolates. All isolates were stimulated to some degree by host tissue extracts. Green cladodes, unsuberized roots, and suberized roots were the most stimulatory, while seeds and senesced cladodes were less stimulatory. The growth of the *Casuarina*-infective *Frankia* strain CjI82 001 was stimulated more than other strains, with 100% increased growth relative to controls with extracts of green cladodes (Figure 1d). The more-pronounced growth stimulation suggests that compounds released from host tissue specifically favor *Casuarina*-infective *Frankia*. Compounds from the host tissue may be a contributing factor in the localization of *Casuarina*-infective *Frankia* in soils near a *Casuarina* host (Zimpfer et al., 1999).

TABLE 5. MEAN GROWTH (PROTEIN ACCUMULATION)[a] OF BACTERIA SPECIES WITH AQUEOUS EXTRACTS OF *Casuarina cunninghamiana* CLADODES ADDED TO THE MEDIUM

Bacteria species	Hours of growth	Cladode concentration (g/l)			
		0	2	5	10
Streptomyces albus	0	1.4	1.4	1.4	1.4
	2	10.8a[b]	8.3ab	13.5a	4.7b
	6	15.5	11.2	13.7	10.2
	12	38.6	52.8	42.8	45.8
	24	98.3c	136.4b	178.6a	180.3a
	48	244.8	242.4	246	244.6
Bradyrhizobium japonicum	0	0.1	0.1	0.1	0.1
	4	10.8a	3.6b	.6c	.8c
	8	20.4a	6.8b	0.6c	1.0c
	12	35.4a	13.3b	20.5b	17.0b
	24	50.1a	41.2b	21.8d	32.9c
	48	70.7a	62.0ab	58.2b	72.0a
Bacillus subtillis	0	0.5	0.5	0.5	0.5
	2	11.2a	1.8b	11.0a	4.3ab
	12	15	15.5	14.6	13.9
	24	41.1a	40.1a	32.9ab	30.7a
	48	69.2ab	78.7a	70.4ab	57.6b
Arthrobacter globiformis	0	0.1	0.1	0.1	0.1
	2	2.6b	2.8b	5.9a	5.3a
	4	10.8a	2.9b	12.3a	10.6a
	8	35.2c	38.8bc	62.6a	50.2ba
	12	94.8	75.9	80.8	76
	24	113.8a	89.92b	93.2ab	93.6ab

Note. Bacteria were grown in glass tubes with 10 ml of liquid medium ($N = 3$).

[a] Microgram protein ml^{-1} medium.

[b] Where there were significant differences in growth between tissue concentrations in an ANOVA, means in a row followed by the same letter are not significantly different ($P < 0.05$).

Over time, with differing concentrations of aqueous extracts of *C. cunninghamiana* cladodes, both tested strains of *Frankia* isolated from *Casuarina* were stimulated to a greater degree than were other soil microorganisms, with the exception of *S. albus*, another actinomycete. After 3 d growth, cultures of *Frankia* strain CjI82 001 with a 2 g/l cladode tissue extract concentration showed an increase in growth of more than 400% compared to 3 d old control cultures. We did not observe an increase in growth of *Frankia* strain CjI82 001 when aqueous extracts of *T. catappa* were added, suggesting that increases in growth do not result from a general response to plant tissue. *C. cunninghamiana* cladode extracts inhibited or failed to stimulate non actinomycetous bacteria. *Frankia* strain 55005, an atypical *Casuarina*-derived isolate that does not infect its host, was the only bacterial isolate to be stimulated by the highest concentration of cladode extracts (Table 4).

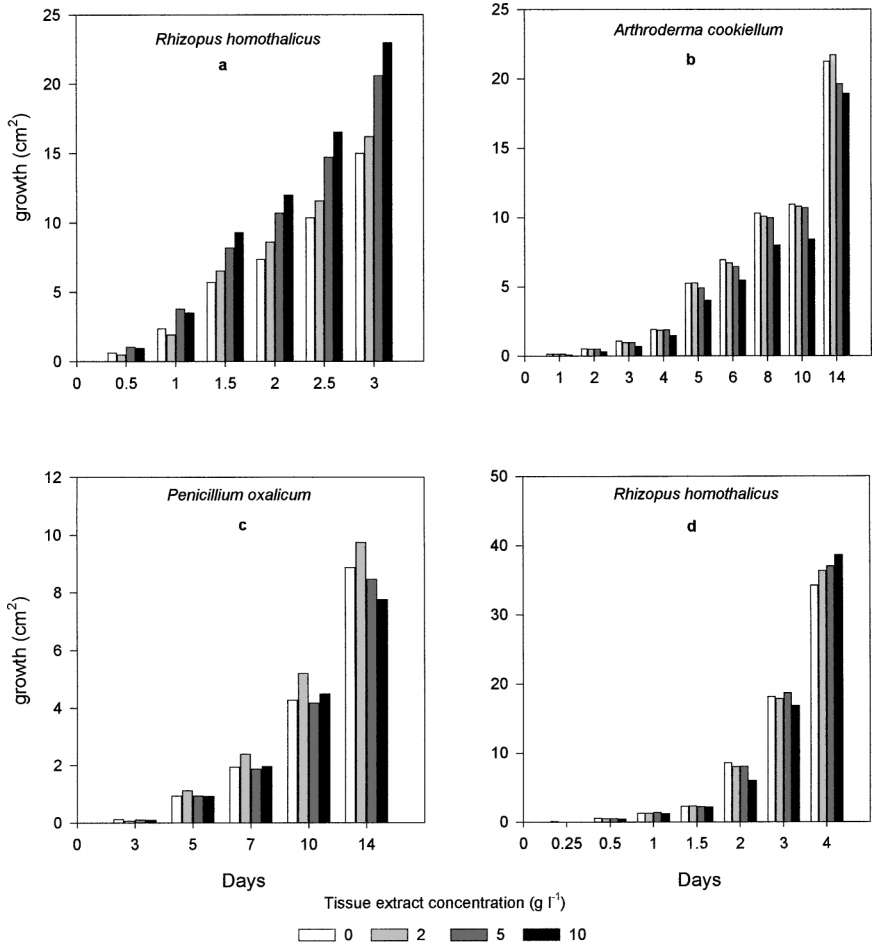

FIG. 2. Cumulative area growth of fungal colonies on agar nutrient media containing varying concentrations of *Casuarina cunninghamiana* cladode (a, b, c) or *Terminalia catappa* leaf (d) extracts. Repeated measures analysis of variance ($P < 0.01$) indicates significant growth increase (a), significant growth inhibition (b), and no significant growth difference (c, d) over time with increasing concentrations of plant extracts.

Perhaps, this strain exists in host rhizospheres and possesses either an enhanced tolerance of, or greater ability to, metabolize host secondary compounds.

There was not a consistent trend in the influence of extracts of *C. cunninghamiana* on soil fungi. Growth of *R. homothallicus* was stimulated by *C. cunninghamiana* cladodes but not by *T. catappa* leaves, indicating that the stimulation in growth was not a result of compounds present in all plant tissue. Additionally, with

the fresh weight equivalent of 10 g/l of *C. cunninghamiana* cladode tissue added to agar media, the rate of growth of *A. cookiellum* decreased significantly, and the region of the growing front became dark red in color. Discoloration of the agar was identical to that of the growing fronts of colonies, of *A. cookiellum* converging with *Penicillium* sp. colonies, suggesting that extracts of *C. cunninghamiana* possess antibiotic properties.

Casuarina trees may influence the composition of the soil microbial community in their rhizospheres through the release of compounds from living tissue. Elo et al. (2000) determined that silver birch and fescue grass selectively attracted specific bacteria to their rhizoplanes. Sayed and Wheeler (1999) found that the flavonoid quercetin added to isolation media increased the number of *Frankia* colonies and decreased fungal contaminants. Janczarek et al. (1996) observed that commercial flavonoids and clover exudates increased the rate of growth of *Rhizobium leguminosarum* bv. *Trifolii* strains. Phenolics of plant tissue (Harborne, 1973) and soils (Whitehead, 1964; Li et al., 1970; Whitehead et al., 1983) may influence the interaction between plants and soil microorganisms. Perradin et al. (1983) and Vogel and Dawson (1986) found that phenolics could significantly stimulate or inhibit the *in vitro* growth of *Frankia* as well as the morphological development of *Frankia*, according to the specific phenolic compound and *Frankia* strain tested.

Stimulation of soil respiration by *Casuarina* extracts (Zimpfer et al., 2003) suggests that an overall inhibition of the soil microbial community is not occurring with the addition of *Casuarina* host tissue extracts. There remains the possibility that a selective inhibition of specific microbial taxa occurs. Concurrent increases in soil infective capacity suggest that other members of the soil microbial community may be important in the persistence of *Frankia* in the soil or the nodulation process itself. Knowlton and Dawson (1983) found that *Frankia* coinoculation with *Pseudomonas cepacia* increased nodule numbers on alder seedlings grown under axenic conditions. Similarly, we have observed axenic cultures of *Casuarina* seedlings and infective *Frankia* on agar slants to nodulate only when contaminated (unpublished data). If *Casuarina* were able to favor beneficial soil microorganism in their rhizospheres and inhibit harmful microoranisms through the release of specific compounds from host tissue, the ecological fitness of this symbiotic pair would be increased.

It is unlikely that our observed stimulation in *Frankia* growth can be attributed to changes in pH, water potential, or osmotic effects. Microorganisms commonly produce metabolites that can modify the pH of media, thus influencing their own growth. However, the media in this study were buffered, so that pH was held within a narrow range. The optimum pH for *Frankia* is around 6.8, the initial pH of the *Frankia* growth medium. Any change in pH would likely have produced some decrease in growth rate that would have counteracted, not amplified the observed stimulation of *Frankia* growth. The contribution of the added tissue

extract to the osmotic potential of all the media is trivial relative to their contained mineral salts and organic components. Five grams of fresh tissue contain about 0.03 g of minerals and lesser concentration of soluble sugars. *Frankia* strains from *Casuarina* tend to be tolerant of the effects of both high salt concentration and negative water potentials and would not be influenced by osmotic changes wrought by the additions of small amounts of plant tissue (Burleigh and Dawson, 1991).

Several *C. cunninghamiana* tissue types contain compounds capable of selectively stimulating the growth of *Frankia* isolates. Compounds present in *C. cunninghamiana* tissue have the ability to stimulate or inhibit the growth of some common soil bacteria and fungi, and possibly alter the species composition of the host rhizosphere. Our results raise the possibility that compounds synthesized by the actinorhizal host plant stimulate the growth of strains of their microsymbiont *Frankia* and microorganisms that are neutral or beneficial to the symbiotic partners, while inhibiting soil microorganisms antagonistic to *Frankia* and its hosts. Insofar as effective quantities of these compounds are released into the rhizosphere, microbial populations favorable to the establishment of symbiotic root nodules could be fostered.

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JUGLONE DISRUPTS ROOT PLASMA MEMBRANE H^+-ATPase ACTIVITY AND IMPAIRS WATER UPTAKE, ROOT RESPIRATION, AND GROWTH IN SOYBEAN (*Glycine max*) AND CORN (*Zea mays*)

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Abstract—Juglone is phytotoxic, but the mechanisms of growth inhibition have not been fully explained. Previous studies have proposed that disruption of electron transport functions in mitochondria and chloroplasts contribute to observed growth reduction in species exposed to juglone. In studies reported here, corn and soybean seedlings grown in nutrient solution amended with 10, 50, or 100 μM juglone showed significant decreases in root and shoot dry weights and lengths with increasing concentrations. However, no significant differences in leaf chlorophyll fluorescence or CO_2-dependent leaf oxygen evolution were observed, even in seedlings that were visibly affected. Disruption of root oxygen uptake was positively correlated with increasing concentrations of juglone, suggesting that juglone may reach mitochondria in root cells. Water uptake and acid efflux also decreased for corn and soybean seedlings treated with juglone, suggesting that juglone may affect metabolism of root cells by disrupting root plasma membrane function. Therefore, the effect of juglone on H^+-ATPase activity in corn and soybean root microsomes was tested. Juglone treatments from 10 to 1000 μM significantly reduced H^+-ATPase activity compared to controls. This inhibition of H^+-ATPase activity and observed reduction of water uptake offers a logical explanation for previously documented phytotoxicity of juglone. Impairment of this enzyme's activity could affect plant growth in a number of ways because proton-pumping in root cells drives essential plant processes such as solute uptake and, hence, water uptake.

Key Words—Juglone, H^+-ATPase, plant water relationships, root plasma membrane, leaf photosynthesis, root respiration, allelopathy, *Glycine max, Zea mays, Juglans nigra.*

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INTRODUCTION

Quinones are ubiquitous molecules that have many physiological roles, including serving as functional constituents of biochemical systems (such as ubiquinone and plastoquinone), dyes or pigmentation, or defensive compounds (Thomson, 1971; Öllinger and Brunmark, 1991). Juglone (5-hydroxy-1,4-naphthoquinone), an amber-colored compound that has been isolated from numerous *Juglans* spp., is one of the more widely studied quinones (Lee and Campbell, 1969; Graves et al., 1979). Duroux et al. (1998) suggest that hydrojuglone, the colorless, nontoxic reduced form found in the living tissue, may take part in plant developmental processes and defense mechanisms.

When exposed to air, hydrojuglone is immediately oxidized to its purportedly toxic form, juglone (Rietveld, 1983). Some toxic effects attributed to juglone include inhibitory effects on insect larval development and insect flight muscle mitochondria, sedative effects on fish and animals, mutagenic, carcinogenic, and lethal effects on animal cells, and antimicrobial, antifungal, and antiparasitic activity (Westfall et al., 1961; Van Duuren et al., 1978; Öllinger and Brunmark, 1991; Zhang et al., 1994; Magiri et al., 1995; Weissenberg et al., 1997; Cai et al., 2000).

Juglone has been widely studied for allelopathic effects ever since Massey (1925) hypothesized that a compound exuded by the roots was a likely cause of the inhibitory effect of black walnut (*Juglans nigra* L.) on growth of some associated species. Davis (1928) attributed that toxicity to an exudate he identified as juglone. In the literature, some reports dispute how long juglone may persist in the soil and the extent to which effects on neighboring species can be attributed to phytotoxicity rather than other factors such as resource competition (Ponder and Tadros, 1985; Schmidt, 1988; De Scisciolo et al., 1990; Seifert, 1991; Weidenhamer, 1996; Jose and Gillispie, 1998a). However, growth inhibition at micromolar concentrations has been reported for numerous plant species (Brooks, 1951; MacDaniels and Pinnow, 1976; Funk et al., 1979; Dawson and Seymour, 1983; Rietveld, 1983; Rietveld et al., 1983; Jose and Gillispie, 1998b).

In striving to reveal mechanisms of plant growth reduction by juglone, studies have demonstrated impairment of root, leaf, and cotyledon respiration (Perry, 1967; Koeppe, 1972; Peñuelas et al., 1996; Jose and Gillispie, 1998b) and decreased photosynthesis in leaf tissue (Hejl et al., 1993; Jose and Gillispie, 1998b). Increased oxygen uptake in isolated soybean and corn mitochondria and reduced CO_2-dependent oxygen evolution in isolated pea chloroplasts were reported by Hejl et al. (1993) with juglone concentrations as low as 2 μM. Others have shown that juglone inhibits *p*-hydroxyphenylpyruvate dioxygenase (I50 = 1.3 μM), the key enzyme for plastoquinone synthesis (Meazza et al., 2002). However, showing disruption of photosynthesis and respiration in vivo and in vitro does not necessarily confirm that this is a primary or direct mechanism of juglone-mediated growth reduction. Other physiological perturbations (e.g., chronic water stress)

could cause indirect disruption before significant amounts of juglone reach mitochondria or chloroplasts. In fact, because of their high lipophilicity, Fedtke (1993) suggested that quinones may have low activity beyond the root system, and Zhang et al. (1994) proposed that cell membranes might be the main target of juglone toxicity.

Significant inhibition of transpiration and stomatal conductance reported by Jose and Gillispie (1998b) in hydroponically grown corn and soybeans exposed to juglone suggests that this phytotoxin may interfere with normal water transport. Water uptake and transport in plants can be impaired for a variety of reasons, but disruption of root plasma membrane functions could be a factor that would interfere with water uptake and, consequently, a number of plant processes, with widespread detrimental effects on the plant (Glass and Dunlap, 1974; Crane and Möller, 1988). More specifically, impairment of the plasma membrane H^+-ATPase would diminish the ability of the root cells to create pH and electrical potential differences across the plasma membrane, thus causing extensive negative effects on the plant (Briskin and Hanson, 1992; Michelet and Boutry, 1995; Steudle and Peterson, 1998).

To confirm its ability to disrupt growth and energy metabolism, the effects of juglone on shoot and root lengths and dry weights, root respiration (oxygen uptake), and leaf photosynthesis (fluorescence and CO_2-dependent oxygen evolution) in hydroponically grown soybean (*Glycine max* L. Merr.) and corn (*Zea mays* L.) seedlings were measured. ATPase assays were conducted using root microsomal fractions of each species to test the hypothesis that juglone interferes with H^+-ATPase function and consequently reduces proton pumping (Hejl and Koster, 1998). Acidification (ΔpH) of the nutrient solution was measured as additional evidence of the diminished ability of roots to extrude protons in the presence of juglone. Nutrient solution use and relative water content were also determined to test the hypothesis that, as a result of decreased proton pumping by the roots, there is reduced uptake of water after exposure to juglone.

METHODS AND MATERIALS

Germination, Hydroponic Propagation, and Growth Conditions. Hydroponically grown corn and soybeans were propagated and treated according to a procedure modified from Einhellig and Souza (1992). Corn seeds (*Zea mays* L., Cargill 6327F14) and soybean seeds (*Glycine max* L. Merr., Prairie Brand 277) were germinated on moistened germination paper (Anchor Paper Co., St. Paul, MN) at room temperature (23–28°C) under a halogen lamp with a 12 hr photoperiod (250–300 μmol m^{-2} s^{-1} light intensity). Four- to five-day-old seedlings of each species were selected for uniformity of size and transferred to individual opaque growth vials containing 70 ml of a modified Hoagland's (Hoagland and Arnon, 1950) nutrient

solution (5 mM $Ca(NO_3)_2$, 5 mM KNO_3, 2 mM $MgSO_4$, 1 mM KH_2PO_4, 50 μM H_3BO_3, 10 μM $MnCl_2$, 1 μM $ZnCl_2$, 0.3 μM $CuCl_2$, 0.1 μM Na_2MoO_4, 70 μM Fe-EDTA for soybeans or 140 μM Fe-EDTA for corn, pH adjusted to 7.0 with KOH or HCl) and returned to the same growth conditions for 2–3 d acclimation before beginning treatment. Treatments of 0, 10, 50, or 100 μM juglone (Sigma) were used in nutrient solution unless otherwise noted, and fresh nutrient solution with treatment was provided every 3–4 d during the study period to each seedling to ensure that sufficient amounts of ions, unoxidized iron, and water were available. The difference between initial pH and initial volume and the pH and volume of solution remaining were determined at each solution change. Decrease in 2° root growth, nutrient solution uptake, and solution acidification, along with darkening of roots, were noted in juglone treatment groups. A second study was undertaken to measure these parameters daily over the first 4 d of juglone exposure.

Seedling Growth Parameters. Seedlings were harvested when some treatment groups appeared severely affected (after 7 d of treatment for soybeans and 17 d of treatment for corn). Root and shoot lengths were determined to the nearest millimeter (mm) and root and shoot dry weights (DW) to the nearest milligram (mg) (dried 24 hr at 100°C in a forced-air oven). In the 4 d growth experiment, roots were harvested after 1, 2, 3, and 4 d of juglone treatment, root fresh weight (FW) was determined to the nearest mg, and DW was determined as described above. Relative water content (RWC) was calculated according to the following formula (where T = treatment and C = control):

$$\mathrm{RWC} = \left(\frac{(\mathrm{FW_T} - \mathrm{DW_T})/\mathrm{DW_T}}{(\mathrm{FW_C} - \mathrm{DW_C})/\mathrm{DW_C}} \right) \times 100$$

Chlorophyll Fluorescence. Chlorophyll fluorescence was measured every 3–4 d immediately before nutrient solution replacement and after plants were exposed to at least 2 hr of light. The two most fully emerged leaves on each plant were selected and dark adapted for 15 min. Initial fluorescence (F_0) and maximal fluorescence (F_m)were measured using an Opti-Sciences OS-100 modulated fluorometer (PP Systems, Inc. USA). The efficiency of excitation capture was recorded as F_v/F_m and calculated on the fluorometer, which used the formula $F_v = F_m - F_0$ to determine variable fluorescence (F_v).

Leaf CO_2-Dependent Oxygen Evolution. After 8 d of hydroponic growth and juglone treatment, CO_2-dependent oxygen evolution was measured on leaf sections cut from the largest fully-emerged leaf. To standardize leaf area in the chamber for each trial, each leaf was cut with a new razor blade into a 2 × 2 cm square for soybeans and a 3 cm length at the widest part of the leaf for corn. Oxygen evolution was measured at 25°C in an air-phase chamber fixed to a Hansatech Clark-type O_2 electrode (Kings Lynn, Norfolk, England) according to a procedure modified from Delieu and Walker (1981). Each leaf section was laid on a mesh wire disk resting on a sponge soaked with 500 μl of a 1 M $NaHCO_3$ solution as a source of

CO_2. The chamber was sealed and calibrated (nmol O_2 per mV). After a linear rate was established, mV change was recorded for 10–15 min, and O_2 evolution was calculated from the most linear 5 min section of the recorded slope and reported as nmol O_2 cm^{-2} min^{-1}.

Root Oxygen Uptake. Corn and soybean roots were harvested after 4 d of hydroponic growth and juglone treatment. They were rinsed gently in deionized water and blotted dry. Approximately 50 mg of fresh root tissue were cut in 5 mm segments from the apical end of the root with a new razor blade and transferred immediately to an aqueous-phase chamber fixed to a Hansatech Clark-type oxygen electrode (Kings Lynn, Norfolk, England) containing 2 ml of aerated deionized water at 25°C. Change in mV was recorded, and O_2 uptake was calculated and reported as nmol O_2 g FW^{-1} min^{-1}. Root DW was determined so O_2 uptake could also be reported on a dry weight basis.

Isolation of Root Membranes and ATPase Assay. Seeds of corn or soybeans were dark germinated at 28°C on moistened germination paper, and roots were harvested after 3–4 d growth. The microsomal fraction was obtained according to a procedure modified from Calera et al. (1995). Maintaining temperatures of 0–4°C throughout the procedure, approximately 10–15 g root tissue were ground with mortar and pestle in 40–50 ml of grinding buffer containing 250 mM sucrose, 12.5 mM MES-TRIS (pH 7.8), 1.25 mM DTT (dithiothreitol), 3 mM EDTA (ethylene diamine tetraacetic acid), and 1 mM PMSF (phenylmethysulfonyl fluoride). The homogenate was filtered through eight layers of cheesecloth, centrifuged for 12 min at 13,000*g*, decanted, and centrifuged for 45 min at 26,000 *g* to obtain microsomal pellets. The supernatant was discarded and pellets resuspended in buffer containing 250 mM sucrose, 1 mM MES-TRIS (pH 7.6), 1 mM DTT, and 5 μg/ml chymostatin. Membrane protein concentration was determined by a method modified from Peterson (1977) using BSA (bovine serum albumin) as a protein standard. The membrane suspension was stored on ice and used within 2–3 hr.

Root plasma membrane H^+-ATPase activity was assayed according to a method modified from Peterson (1978). Approximately 30–40 μg protein from the microsomal suspension was resuspended in 0.8 ml ATPase assay buffer containing 62.5 mM KCl, 37.5 mM MES-TRIS (pH 6.5), 7.6 mM $MgSO_4$, 1.25 mM Na_2MoO_4, 1.25 mM NaN_3, 100 mM KNO_3, and 3 mM ATP. Na_2MoO_4, NaN_3, and KNO_3, inhibitors of acid phosphatase, mitochondrial ATPase and tonoplast ATPase, respectively, were added to ensure that phosphate release by these enzymes did not confound the results of the assays. In some trials, the plasma membrane inhibitor Na_2VO_4 was added at 200 μM to determine what percentage of the microsomal suspension consisted of plasma membranes. Samples were treated with juglone dissolved in ethanol to obtain final concentrations of 0, 5, 10, 50, 100, 250, 500, or 1000 μM, then were incubated at 38°C for 20 min. The amount of ethanol never exceeded 1% (v/v), a concentration that elicited no significant effect in ethanol-only trials as compared to controls. ATP hydrolysis was stopped with

the addition of 0.64 ml of 10% (w/v) SDS (sodium dodecyl sulfate). Then 2.2 ml of 0.905% (w/v) Na_2MoO_4 in 1.45 N HCl and 0.16 ml of 0.05% (w/v) ANSA (1-amino-2-naphthol-4-sulfonic acid) were added and mixed, and the tubes were allowed to stand for 30 min at room temperature for color development. Inorganic phosphate (P_i) release was determined colorimetrically at 700 nm against a water blank and reported as percent of control. Because juglone is an amber-colored compound, experimental trials with juglone were done only to determine if the compound's color contributed to the absorbance at 700 nm. Juglone did cause a small, but statistically significant, amount of absorbance at concentrations above 250 μM (approximately 1% of control for each 100 μM of juglone). Therefore, the mean absorbance of the juglone controls was subtracted from the experimental data to correct mathematically for the contribution of juglone to the absorbance.

Statistical Analysis. Data were subjected to one-way or two-way ANOVA. Significant differences in one-way ANOVA were identified by Student–Newman–Keuls test (SNK-test, $P < 0.05$). Treatment and days were used as predictor variables in two-way ANOVA. Means comparisons were adjusted with Bonferroni-type adjustment at 0.0077 level of probability to reduce Type I error (Zar, 1996).

RESULTS

Seedling Growth. Corn and soybean seedling shoot and root lengths and dry weights were reduced by all juglone treatments. Soybean was more affected than corn in all growth parameters. After only 7 d of treatment, mean total seedling lengths for soybean were 38, 48, and 48% less than controls for 10, 50, and 100 μM treatments, respectively, whereas mean total seedling lengths for corn were 13, 16, and 21% less than the controls after 17 d of the same treatments (Table 1). A similar pattern was also evident in mean shoot and root lengths of both species and in mean total seedling, shoot, and root dry weights (Table 1). The appearance of the corn and soybean seedlings after exposure to juglone was consistent with the measured growth parameters. Within 7 d of exposure to juglone, reduction of growth was visible in all treatment groups of both species (Figures 1 and 2). Leaves on the treated plants appeared smaller than the corresponding leaves on the control plants, and the treated plants looked generally wilted. Root growth was also visibly reduced in plants exposed to juglone compared to controls. In addition, treated roots showed a brown to black discoloration and decreased secondary root development (Figure 2). This discoloration was visible on the roots of soybean seedlings exposed to the two highest concentrations of juglone within 1 d of treatment, but was evident after 3 d in all treatment concentrations of both species. Also, roots on all seedlings grown in juglone treatment had markedly decreased turgor, with roots of soybean seedlings appearing more severely affected.

TABLE 1. LENGTH (LG) AND DRY WEIGHT (DW) FOR HYDROPONICALLY GROWN CORN AND SOYBEAN SEEDLINGS EXPOSED TO JUGLONE[a]

	Corn		Soybeans	
Juglone (μM)	LG (cm)	DW (mg)	LG (cm)	DW (mg)
Root				
0	25 ± 1.4a	151 ± 26a	19 ± 2.8a	57 ± 9a
10	20 ± 3.8b	152 ± 35a	13 ± 1.7b	34 ± 17b
50	20 ± 2.1b	134 ± 17a	12 ± 1.8b	25 ± 5bc
100	19 ± 2.2b	127 ± 20a	12 ± 1.3b	21 ± 7c
Shoot				
0	27 ± 2.3a	308 ± 47a	21 ± 2.4a	228 ± 30a
10	25 ± 2.7ab	235 ± 48b	11 ± 5.9b	152 ± 49b
50	23 ± 4.3b	179 ± 33c	9 ± 3.7b	132 ± 31b
100	22 ± 3.7b	156 ± 23c	9 ± 1.5b	122 ± 20b
Total				
0	52 ± 2.4a	460 ± 63a	40 ± 3.7a	284 ± 37a
10	45 ± 4.8b	387 ± 78b	25 ± 7.4b	186 ± 66b
50	43 ± 5.1b	313 ± 49c	21 ± 3.5b	157 ± 30bc
100	41 ± 3.0b	282 ± 38c	21 ± 1.3b	142 ± 19c

[a] Means (with SD) within each experiment followed by different letters were significantly different at $P < 0.05$ (one way ANOVA, Student–Newman–Keuls test, $N = 12$). Soybeans were harvested for dry weights after 7 d treatment. Corn was harvested after 17 d treatment.

Chlorophyll Fluorescence and Leaf Oxygen Evolution. Chlorophyll fluorescence parameters (data not shown) generally showed no consistent or significant differences for hydroponically grown corn or soybeans. Those means that showed slight significant differences did not show a consistent pattern relative to increasing juglone concentration or treatment time. The ratio of F_v to F_m did not exceed 0.80 in these studies. This ratio represents the fraction of reaction centers inhibited (p). For uninhibited PSII reaction centers, p approaches zero and $p = 1$ with complete inhibition. This ratio typically is 0.75–0.85 for nonstressed plants (Ögren and Baker, 1985; Peterson et al., 1988; Gleiter and Renger, 1993). After 4 d of juglone treatment, CO_2-dependent oxygen evolution in leaf sections of seedlings of both species also showed no significant differences from controls.

Root Segment Oxygen Uptake. After 4 d of juglone treatment, hydroponically grown corn and soybean root segments had increased oxygen uptake compared to controls for all treatment groups (Table 2). This difference was significant in the 50 and 100 μM treatment groups when calculated as a function of fresh weight. However, this difference was not significant on a dry weight basis even though there was a positive correlation in oxygen uptake with increasing concentration of juglone (Table 2).

Nutrient Solution Uptake, Acidification, and Relative Water Content of Roots. All concentrations of juglone treatment in these experiments significantly impaired

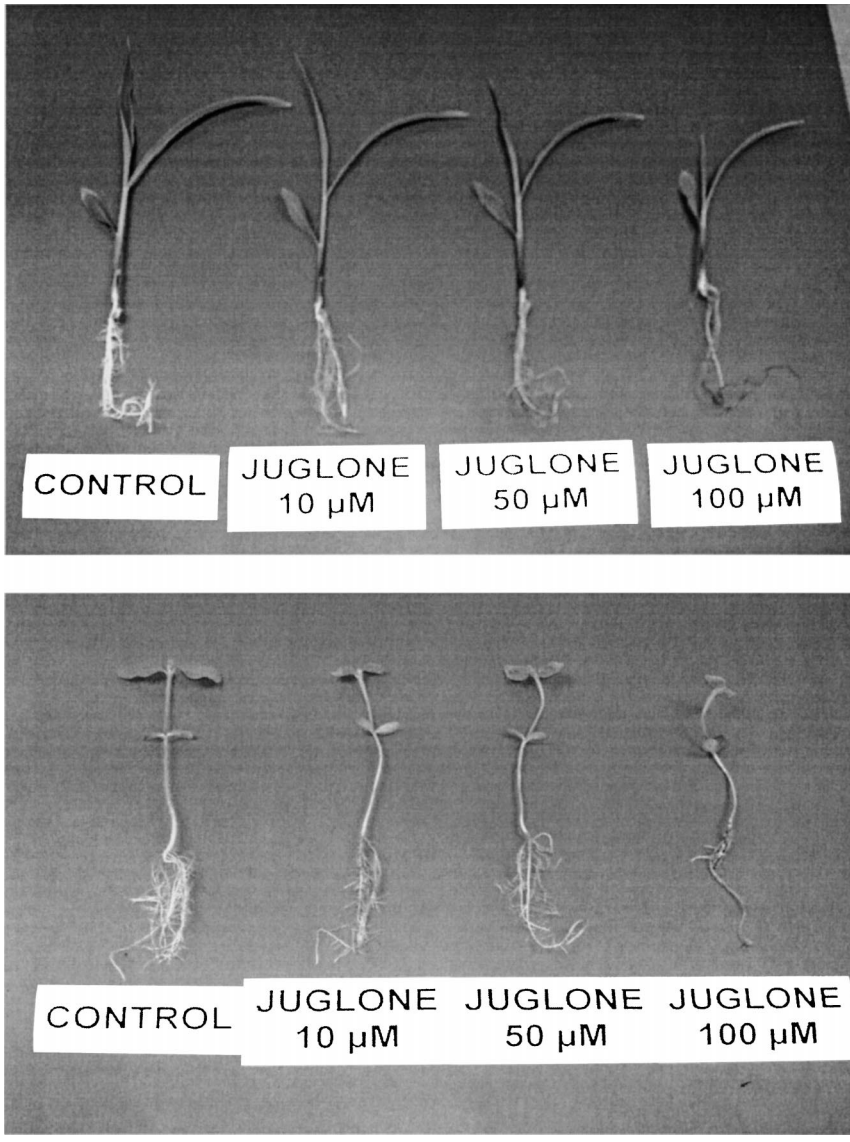

FIG. 1. Appearance of corn (top) and soybean (bottom) seedlings after 7 d treatment with 0, 10, 50, and 100 μM juglone.

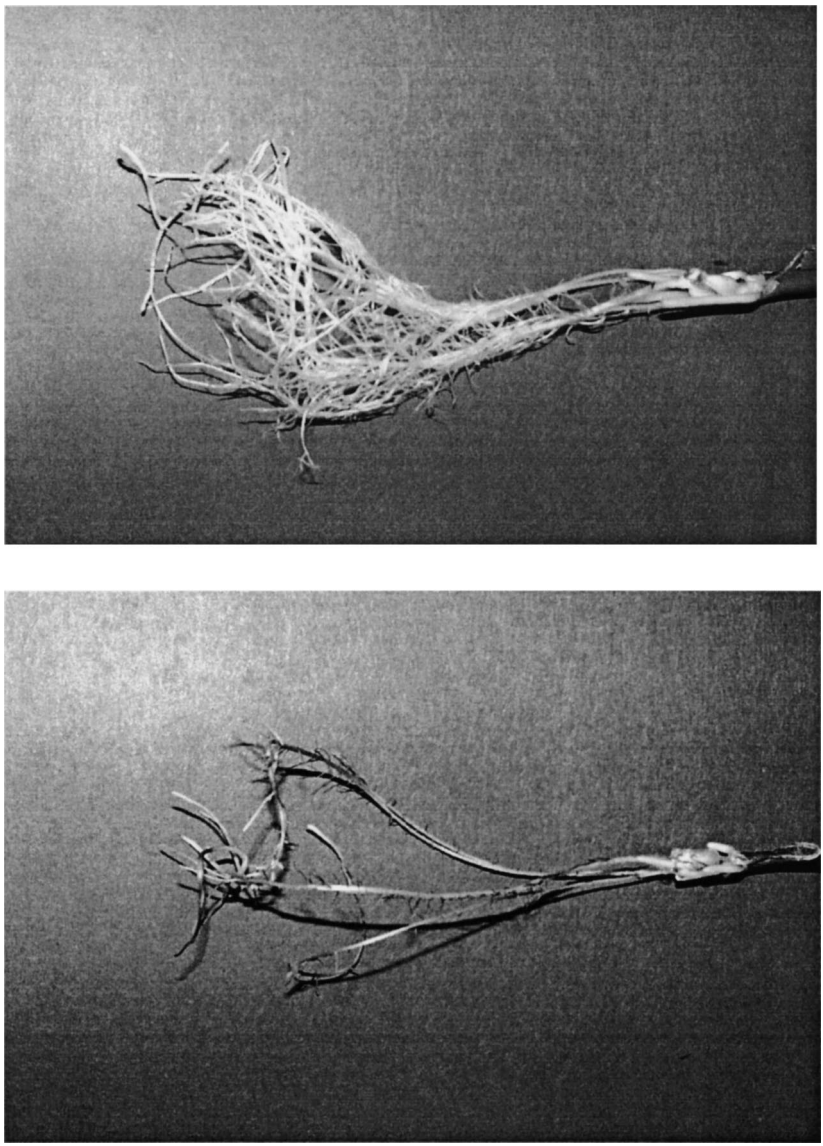

FIG. 2. Appearance of corn roots after 7 d treatment with 0 μM (top) and 100 μM (bottom) juglone.

TABLE 2. ROOT OXYGEN UPTAKE AS A FUNCTION OF ROOT FRESH WEIGHT (FW) AND ROOT DRY WEIGHT (DW) FOR HYDROPONICALLY GROWN CORN AND SOYBEAN EXPOSED TO JUGLONE[a]

	Corn		Soybeans	
Juglone (μM)	μmol O_2/g FW/hr	μmol O_2/g DW/hr	μmol O_2/g FW/hr	μmol O_2/g DW/hr
0	10 ± 1a	204 ± 28a	18 ± 4a	378 ± 78a
10	14 ± 3a	240 ± 55a	19 ± 2ab	392 ± 33a
50	18 ± 1b	249 ± 28a	23 ± 5b	396 ± 73a
100	19 ± 3b	267 ± 56a	25 ± 4b	397 ± 32a

[a] Seedlings were exposed to juglone 4 d. Means (with SD) within each experiment followed by different letters were significantly different at $P < 0.05$ (one way ANOVA, Student–Newman–Keuls test, $N = 3$–6).

nutrient solution uptake by both corn and soybeans, with soybeans showing larger differences among treatments (Tables 3 and 4). By the fourth day of treatment, mean solution uptake per seedling for soybeans in the 10 μM treatment group was only about half that of control values, and in the 50 and 100 μM treatment groups, solution use was less than a third of control uptake. By the seventh day of treatment, solution uptake by soybeans in the 50 and 100 μM treatment groups was negligible. Solution uptake by corn was also significantly decreased, but to a lesser extent, and treated corn seedlings exhibited some recovery, such that solution uptake in later intervals was not reduced as much relative to control seedlings as it was in the initial 0–4 d treatment period (Table 3). Effects of juglone on nutrient solution uptake were particularly apparent in calculations of cumulative uptake (Table 3) with reductions in soybeans of 65, 87, and 90% and 30, 52, and 64% in corn at treatments of 10, 50, and 100 μM, respectively.

When trials were repeated to determine juglone's effects on solution uptake in each of the initial 4 d of treatment, diminished solution use was measurable after 1 d and became significant after 2 d for both species (Table 4). A decrease in relative water content in roots of both species was also measurable after 1 d and was significant each day thereafter for both species, again with greater differences in soybean (Table 5).

Determination of nutrient solution acidification (ΔpH) revealed decreased ability of roots in both species and all treatment groups to acidify the surrounding solution (Tables 3 and 4). As with solution uptake, differences in ΔpH were measurable and significant in the two highest treatment concentrations after 1 d juglone treatment and significant for all concentrations of juglone after 2 d treatment. Soybeans were much more affected than corn, with less acidification of the nutrient solution than corn at all treatment concentrations after only 1 d of exposure to juglone. Corn showed some recovery in the ability of the roots to acidify the solution after the initial 4 d treatment (Table 3).

TABLE 3. NUTRIENT SOLUTION UPTAKE AND ACIDIFICATION (ΔpH) FOR HYDROPONICALLY-GROWN CORN AND SOYBEAN EXPOSED TO LONG-TERM JUGLONE TREATMENT[a]

	Corn		Soybeans	
Juglone (μM)	Solution use (ml)	ΔpH	Solution use (ml)	ΔpH
0–4 d treatment				
0	18 ± 4a	0.8 ± 0.05a	23 ± 7a	1.1 ± 0.18a
10	11 ± 2b	0.6 ± 0.14b	13 ± 7b	0.8 ± 0.37b
50	8 ± 2c	0.5 ± 0.04c	8 ± 4c	0.5 ± 0.08c
100	5 ± 2d	0.3 ± 0.04d	6 ± 3c	0.4 ± 0.05c
4–7 d treatment				
0	20 ± 2a	0.9 ± 0.08a	43 ± 10a	1.0 ± 0.15a
10	14 ± 3b	0.9 ± 0.12a	10 ± 18b	0.5 ± 0.33b
50	9 ± 2c	0.7 ± 0.05b	1 ± 2c	0.4 ± 0.24b
100	8 ± 2c	0.6 ± 0.08c	1 ± 2c	0.2 ± 0.15c
7–11 d treatment				
0	25 ± 2a	1.0 ± 0.11a		
10	21 ± 6b	0.9 ± 0.04b		
50	13 ± 3c	0.7 ± 0.05c		
100	10 ± 2d	0.7 ± 0.05d		
11–14 d treatment				
0	12 ± 2a	0.8 ± 0.14a		
10	8 ± 4b	0.8 ± 0.11a		
50	5 ± 1c	0.6 ± 0.04b		
100	3 ± 1d	0.6 ± 0.08b		
14–17 d treatment				
0	8 ± 5a	0.8 ± 0.11a		
10	5 ± 3b	0.7 ± 0.10b		
50	4 ± 4b	0.6 ± 0.04b		
100	4 ± 4b	0.7 ± 0.05b		
Cumulative Solution Uptake				
0	82 ± 5a		66 ± 10a	
10	58 ± 13b		23 ± 19b	
50	39 ± 6c		9 ± 5c	
100	29 ± 5d		7 ± 4c	

[a] Means (with SD) within each experiment followed by different letters were significantly different at $P < 0.05$ (one way ANOVA, Student–Newman–Keuls test, $N = 12$). Solution uptake was over indicated period with cumulative uptake also shown.

Microsomal H^+-ATPase. Decreased H^+-ATPase activity in root microsomal membranes of both species was apparent at 5 μM juglone and became significant at concentrations $\geq$10 μM (Figure 3). After values were corrected for the color of juglone controls, 1000 μM juglone caused inhibition of P_i release equal to that of 200 μM sodium vanadate.

TABLE 4. NUTRIENT SOLUTION UPTAKE AND ACIDIFICATION (ΔpH) FOR HYDROPONICALLY GROWN CORN AND SOYBEAN EXPOSED TO SHORT-TERM JUGLONE TREATMENTS[a]

	Corn		Soybeans	
Juglone (μM)	Solution use (ml)	ΔpH	Solution use(ml)	ΔpH
1 d treatment				
0	2.1 ± 0.8a	0.7 ± 0.15a	2.0 ± 0.5a	0.7 ± 0.13a
10	1.5 ± 0.5ab	0.6 ± 0.11ab	1.4 ± 0.5ab	0.4 ± 0.08b
50	1.0 ± 0.5b	0.5 ± 0.10bc	1.4 ± 0.5ab	0.4 ± 0.05b
100	1.1 ± 0.6b	0.5 ± 0.05c	0.9 ± 0.3b	0.4 ± 0.05b
2 d treatment				
0	5.1 ± 0.6a	1.3 ± 0.30a	5.2 ± 0.7a	1.0 ± 0.13a
10	3.9 ± 0.6b	0.9 ± 0.21b	3.1 ± 0.6b	0.5 ± 0.20b
50	3.0 ± 0,5c	0.7 ± 0.13c	2.9 ± 0.8b	0.3 ± 0.11c
100	2.1 ± 1.0d	0.6 ± 0.11c	2.0 ± 0.5c	0.4 ± 0.13c
3 d treatment				
0	8.5 ± 1.4a	1.8 ± 0.25a	7.8 ± 1.1a	1.3 ± 0.15a
10	7.4 ± 0.7a	1.4 ± 0.27b	5.2 ± 1.0b	0.9 ± 0.14b
50	5.6 ± 1.2b	0.8 ± 0.12c	4.2 ± 0.7b	0.4 ± 0.17c
100	4.3 ± 1.0c	0.6 ± 0.14c	4.3 ± 0.7b	0.3 ± 0.12c
4 d treatment				
0	11.9 ± 2.2a	1.9 ± 0.22a	13.1 ± 1.5a	1.9 ± 0.46a
10	9.3 ± 0.9b	1.4 ± 0.32b	8.2 ± 1.2b	1.0 ± 0.26b
50	5.9 ± 1.1c	0.7 ± 0.16c	6.2 ± 1.0c	0.4 ± 0.21c
100	4.8 ± 07c	0.5 ± 0.16d	4.9 ± 0.9d	0.2 ± 0.23c

[a] Means (with SD) within each experiment followed by different letters were significantly different at $P < 0.05$ (one way ANOVA, Student–Newman–Keuls test, $N = 8$–9).

DISCUSSION

Our findings are consistent with those of Jose and Gillispie (1998b) who reported significant reductions in relative growth rates of shoots and roots of hydroponically grown corn and soybeans over a 3 d growth period when exposed to 10 and 100 μM juglone. They also found soybeans to be more affected than corn. Reduced growth of various herbs, shrubs, coniferous seedlings, and deciduous trees has been reported at similar concentrations (Funk et al., 1979; Rietveld, 1983). Hejl et al. (1993) demonstrated that juglone can interfere with electron transport in isolated chloroplasts and mitochondria, and they suggested that disruptions of photosynthesis and respiration could account for observed reductions in growth. This shows that juglone does interfere with the processes of photosynthesis and respiration *in vitro*, whereas studies demonstrating that juglone gets to the chloroplasts and mitochondria to interrupt electron transport are lacking, and results of experiments reported here do not indicate a direct effect *in vivo.*

TABLE 5. ROOT FRESH WEIGHT (FW), DRY WEIGHT (DW), AND RELATIVE WATER CONTENT (RWC) FOR HYDROPONICALLY GROWN CORN AND SOYBEAN EXPOSED TO JUGLONE[a]

	Corn			Soybeans		
Juglone (μM)	FW (mg)	DW (mg)	RWC	FW (mg)	DW (mg)	RWC
1 d treatment						
0	197 ± 85a	13 ± 5a	100 ± 17a	246 ± 52a	16 ± 3a	100 ± 6a
10	166 ± 63a	14 ± 5a	80 ± 11b	180 ± 50b	14 ± 4a	86 ± 5b
50	133 ± 44a	12 ± 3a	74 ± 10b	145 ± 31b	11 ± 2b	84 ± 8b
100	101 ± 34b	9 ± 3a	79 ± 11b	138 ± 41b	11 ± 3b	79 ± 9b
2 d treatment						
0	348 ± 62a	20 ± 4a	100 ± 13a	380 ± 60a	30 ± 4a	100 ± 3a
10	232 ± 69b	16 ± 4ab	82 ± 8b	210 ± 48b	17 ± 3b	72 ± 10b
50	165 ± 51c	13 ± 4b	70 ± 11b	167 ± 43bc	15 ± 2b	65 ± 10b
100	154 ± 40c	12 ± 3b	69 ± 7b	128 ± 30c	11 ± 2c	66 ± 13b
3 d treatment						
0	434 ± 98a	23 ± 6a	100 ± 15a	477 ± 127a	29 ± 6a	100 ± 6a
10	332 ± 75b	18 ± 3ab	95 ± 13a	312 ± 112b	24 ± 7ab	76 ± 12b
50	207 ± 48c	16 ± 4b	68 ± 5b	181 ± 64c	18 ± 6bc	58 ± 14c
100	155 ± 51c	12 ± 3b	67 ± 8b	164 ± 43c	15 ± 4c	65 ± 8c
4 d treatment						
0	670 ± 205a	32 ± 9a	100 ± 6a	707 ± 146a	37 ± 6a	100 ± 5a
10	438 ± 147b	22 ± 6b	93 ± 11a	349 ± 87b	25 ± 5b	69 ± 12b
50	184 ± 55c	13 ± 4c	65 ± 8b	230 ± 67c	19 ± 4c	62 ± 11bc
100	123 ± 36c	10 ± 3c	54 ± 6c	182 ± 71c	15 ± 5c	58 ± 10c

[a] Means (with SD) within each experiment followed by different letters were significantly different at $P < 0.05$ (one way ANOVA, Student–Newman–Keuls test, $N = 8$–9).

Although chlorophyll fluorescence and CO_2-dependent oxygen evolution experiments reported here did not show significant differences from controls, Jose and Gillispie (1998b) measured whole leaf photosynthesis using an infrared gas analyzer and demonstrated a modest, but significant, reduction of net photosynthetic rates in leaves of hydroponically grown corn and soybeans after 3 d of juglone treatment at 10 and 100 μM concentrations. Jose and Gillispie (1998b) also showed that the same concentrations of juglone significantly reduced transpiration and stomatal conductance, and they suggested that indirect inhibitory effects on photosynthesis were likely. Our data, showing no significant decrease in photosynthesis in leaf segments detached from juglone-treated plants, may be reconciled to those of Jose and Gillispie (1998b) if it is considered that the detached leaf segments might have open stomata in the high light and humidity of the O_2-electrode chamber. In contrast, the stomata on the intact, attached leaves used by Jose and Gillispie (1998b) were still subject to the inhibitory effects of low plant water potential, as they demonstrated. In combination, these observations suggest

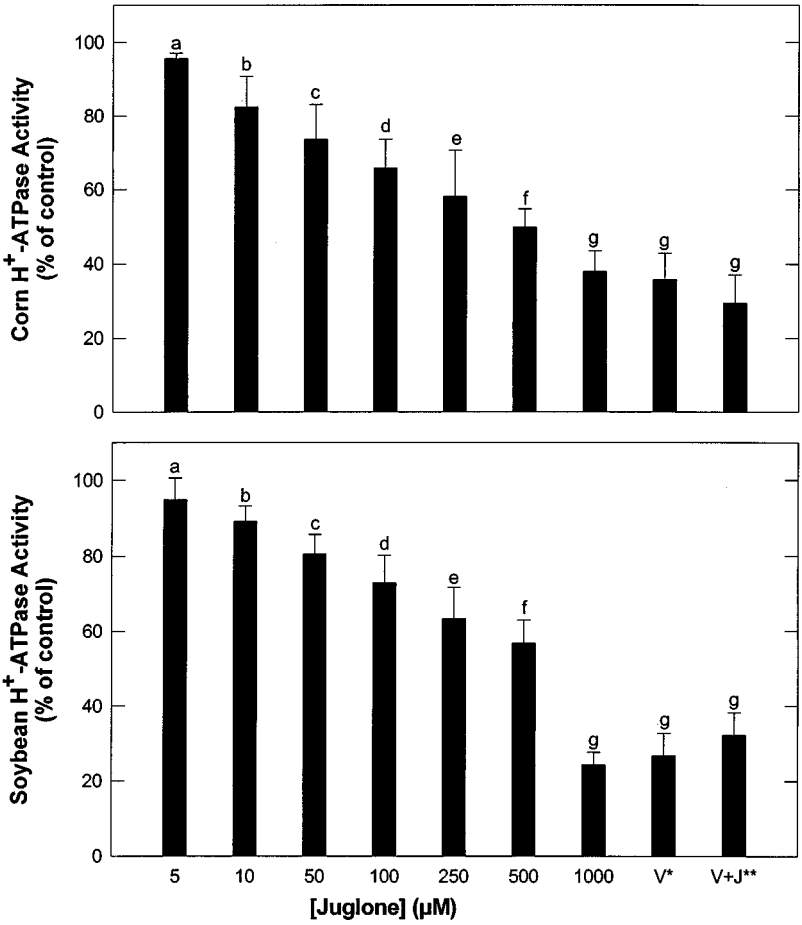

FIG. 3. Influence of juglone on H^+-ATPase activity in corn and soybean root microsomal fractions. Error bars represent one standard error of the mean. Bars with different letters are significantly different at $P < 0.05$ (Student–Newman–Keuls test, $N = 5$–7). $^*V = 200\ \mu$M sodium vanadate. $^{**}V + J = 200\ \mu$M sodium vanadate + 250 μM juglone.

that the chloroplasts themselves were not impaired by treatment of the roots with juglone, but that photosynthesis in the intact plant was reduced by stomatal closure resulting from decreased water uptake by the roots.

Increased oxygen uptake per gram FW by corn and soybean roots in response to increasing concentrations of juglone is consistent with previous findings showing increased oxygen uptake for isolated mitochondria (Koeppe, 1972; Hejl et al., 1993). However, when the data were compared on a DW basis to correct for the

different RWC of the treated roots, no significant differences were found (Table 2). In contrast, Jose and Gillispie (1998b) measured root respiration on the entire intact root system using an infrared gas analyzer and reported small, yet significant, reductions on a DW basis in root respiration, with a maximum reduction of 27% for corn and 52% for soybeans with juglone treatment of 100 μM. Reduction in leaf respiration of various species has been reported (Perry 1967; Jose and Gillispie 1998b), but again, stomatal closure could be a reasonable cause.

In the experiments reported here, significant decreases by treated corn and soybean seedlings in root RWC and in the ability of roots to acidify and use nutrient solutions were observed after as little as 1 d of exposure to juglone. Furthermore, a decrease in H^+-ATPase activity was positively correlated with increasing juglone concentration in corn and soybean root microsomal membranes. These data support the hypothesis that juglone-mediated reductions in growth arise from the decreased ability of the roots to translocate water secondary to inhibition of plasma membrane H^+-ATPase activity. These data also were corroborated by observations that seedlings appeared wilted, like drought-affected plants, and the roots appeared flaccid, even though submerged in nutrient solution. The plant cell plasma-membrane H^+-ATPase and associated membrane proteins play an essential role in the maintenance of cell turgor and uptake of components essential for growth (Babakov et al. 2000). Significant reduction in mineral and water uptake by roots subsequent to H^+-ATPase inhibition in root cells would lead to closing of stomata and have a strong indirect effect on numerous essential plant functions, such as respiration, photosynthesis, and protein synthesis, resulting in decreased growth.

A number of studies report allelochemical-induced disruption of plant water balance and perturbations in ion uptake in roots, and some suggest that these observations are due to interference with normal membrane function and disruption of active transport (Glass and Dunlap, 1974; Barkosky et al., 1999, 2000). Barkosky et al. (1999) concluded that disruptions in water relations appear to be the primary mode of action that leads to overall reductions of growth in leafy spurge in the presence of the phytotoxin hydroquinone.

Likewise, inhibition of enzyme activity is a fairly common herbicide action (Einhellig, 1995), and many herbicides have been shown to inhibit plasma membrane H^+-ATPase (Tu et al., 1995; Hull and Cobb 1998). A number of other phytotoxic substances from various sources have been shown to have an inhibitory effect on plasma membrane H^+-ATPase activity. Hydrophobic substances called betacolins, isolated from the fungus *Cercospora beticola*, inhibited H^+-ATPase activity in plasma membranes isolated from corn roots (Blein et al., 1988). The bacterial phytotoxins syringomycin, syringostatin, and syringotoxin from *Pseudomonas syringae* pv. *syringae* inhibit H^+-ATPase activity in plasma membrane vesicles isolated from corn and mung bean (*Vigna radiata*) at concentrations greater than 10 μM (Che et al., 1992; Di Giorgio et al., 1994). Friebe et al. (1997) also reported decreased H^+-ATPase activity in plasma membrane vesicles

isolated from oat (*Avena sativa*) and vetch (*Vicia faba*) roots upon exposure to two allelopathic compounds from the *Poaceae*, 2,4-dihydroxy-1,4-benzoxazin-3-one and 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one, and they correlated the inhibition of H^+-ATPase activity to reduced radicle elongation in oat seedlings treated with these phytotoxins.

Differences between corn and soybean were not apparent in assays of H^+-ATPase activity (Figure 3), although the species differed in growth and solution uptake. Friebe et al. (1997) cautioned that multiple interactions with numerous physiological and biochemical events must be considered when investigating phytotoxins; this would also be appropriate for the data reported here. It has been suggested that corn is more resistant to some stresses, including effects of drought and herbicides, so corn may simply be resistant to the effects of juglone, as reported for some other grasses (Funt and Martin, 2000). Sparla et al. (1999) isolated a flavoreductase from corn seedlings, which was capable of reducing both ferric chelates and quinones and thus might, detoxify quinones such as juglone. This idea is further supported by the partial recovery of solution acidification and uptake observed in corn seedlings exposed to juglone treatment greater than 4 d in duration (Table 5).

Because of decreased solution uptake and acidification by both corn and soybean seedlings in the presence of juglone, our primary concern was with the effects of juglone on plasma membrane H^+-ATPase activity. Another possibility to consider is that juglone, being a strong electron acceptor, may inhibit plasma membrane redox systems differentially in the two species. Plant plasma membranes contain redox systems able to transfer electrons to extracellular electron acceptors; these are believed to be involved in elongation growth, cell division, radicle production, and membrane energization (Morré et al., 1988; Döring et al., 1992a; Lüthje et al. 1998). Vitamin K_3, a compound structurally similar to juglone, has been shown to inhibit the activity of the plasma membrane redox system in corn roots (Döring et al., 1992b).

In summary, the data demonstrating reduced H^+-ATPase activity in the presence of juglone provide a possible physiological mechanism for decreased relative water content and decreased ability of corn and soybean roots to acidify and use nutrient solutions when exposed to this phytotoxin. The consequential failure to establish the proton gradient that drives solutes and water uptake by the root cells offers at least a partial explanation for the documented impairment of physiological functions and growth by juglone.

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STEREOCHEMISTRY OF HOST PLANT MONOTERPENES AS MATE LOCATION CUES FOR THE GALL WASP *Antistrophus rufus*

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Abstract—In spring, adult males of the gall wasp *Antistrophus rufus* L. emerge from inconspicuous galls in stems of their host plant *Silphium laciniatum* L. and search for sites on stems where females will later emerge. The behavior of males suggests that they use olfaction rather than visual or tactile cues in searching for mates. In an earlier publication, we reported that galls of *A. rufus* were associated with changes in enantiomeric ratios of α- and β-pinene emitted by plant stems, and hypothesized that monoterpene stereochemistry served as a mate location cue for adult males. Here, we support this hypothesis with bioassays that demonstrate that males can discriminate between galled and ungalled stems, as well as between blends of synthetic monoterpenes with ratios of enantiomers representative of galled and ungalled stems.

Key Words—Asteraceae, *Silphium*, Cynipidae, prairie, pinene, enantiomer.

INTRODUCTION

Larvae of *Antistrophus rufus* L. (Hymenoptera: Cynipidae) feed within ellipsoid galls (~3-mm long) in flowering stems of *Silphium laciniatum* L., an asteraceous prairie perennial (Gillette, 1891; Tooker et al., 2002, 2004; Tooker and Hanks, 2004a,b). Unlike most galling insects, development of *A. rufus* galls does not alter the physical structure of the stem surface (Gillette, 1891; Tooker and Hanks, 2004b). Adult males emerge before females in spring and locate areas on dead stems of host plants, in the apparent absence of visual or tactile cues, where females will later emerge (Tooker et al., 2002). Males defend these diminutive

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territories, driving off other males by charging and head butting (Tooker and Hanks, 2004b).

Antistrophus rufus males display characteristic behaviors while searching for mates on plant stems, turning frequently, lowering their heads, and rapidly drumming their antennae on the stem surface (Tooker et al., 2002; Tooker and Hanks, 2004b), suggesting that males use olfactory cues to detect females in stems. Ratios of monoterpenes emitted by dead stems of *S. laciniatum* included α-pinene, β-pinene, and camphene, but these compounds were produced in similar amounts by galled and ungalled stems (Tooker et al., 2002). Enantiomeric ratios [+:−] of both α- and β-pinene, however, approximated 50:50 in ungalled stems but were significantly skewed in both directions in galled stems (camphene was 100% [−] in all stems; Tooker et al., 2002). Skewness of enantiomeric ratios in both directions suggests that oviposition by gall wasps, or feeding by the larvae, alters in some non-uniform way the expression of genes that code for pinene synthases, in some cases activating or repressing the gene for the synthase of one enantiomer, in other cases influencing the gene for the synthase of the other enantiomer. In bioassays, males responded positively to a blend of synthetic compounds with enantiomeric ratios representative of galled stems (Tooker et al., 2002). Based on these findings, we hypothesized that ratios of pinene enantiomers, altered during development of gall wasp larvae, provide olfactory cues that adult males later use in detecting mates in dead stems of host plants (Tooker et al., 2002).

In this article, we present further support for our hypothesis with bioassays that confirm that adult male *A. rufus* can discriminate between plant stems that contain galls and those that do not, and between blends of synthetic compounds representative of galled and ungalled stems.

METHODS AND MATERIALS

Response to Galled and Ungalled Stems. To determine whether male gall wasps can discriminate between galled and ungalled stems, we conducted a choice test based on a Y-configuration of stems (see Tooker et al., 2002; Tooker and Hanks, 2004b). A dead stem (20-cm long) of the non-host plant *Solidago altissima* L. served as the base of a "Y," and the arms were 20-cm sections of galled and ungalled stems of *S. laciniatum* of approximately equal diameter. The "Y" was positioned on an incline directed toward a north-facing window. Wasps released at the base of the "Y" invariably walked uphill where they encountered the junction of galled and ungalled stems. Wasps "responded" to a stem by remaining on it for at least two minutes; individuals that did not respond to either stem within five min were recorded as "no response." We repeated this bioassay until 38 individuals had responded ($N = 42$ trials), replacing stems and switching galled and ungalled treatments between arms after every five wasps to control for location effects.

We used the X^2 goodness-of-fit test (Sokal and Rohlf, 1995) to test differences between treatments in numbers of responding wasps.

For our bioassays, we collected dead stems of *S. laciniatum* that contained diapausing larvae of *A. rufus* from Fithian Railroad Prairie (FRP, Vermilion Co., IL; Tooker and Hanks, 2004c) and dead stems of the same species that did not contain galls from a prairie garden where *A. rufus* was absent (Lincoln Bookbindery Prairie Garden, Champaign Co., IL). Stems were collected in February 2003. We reared all gall wasps from half of the FRP stems in an incubator (22°C, 16:8 L:D) to eliminate the potential complication of volatile compounds produced by wasps themselves. Thus, the "galled stems" referred to below contained recently abandoned *A. rufus* galls, but no insects. In an unheated outbuilding, we stored ungalled stems and the remaining FRP stems from which we reared adult gall wasps for bioassays.

Response to Synthetic Compounds. To test the response of adult male *A. rufus* to synthetic monoterpenes, we created blends of α- and β-pinene and camphene (Aldrich Chemical Co., Milwaukee, WI) in reagent grade hexane in concentrations of 0.9-ng/ml ($\sim$1 equivalent of a 20-cm stem section in 3-ml). To account for skewness in enantiomeric ratios in both directions from 50:50 that was characteristic of galled stems (see Tooker et al., 2002), we conducted two trials: one with enantiomers of α- and β-pinene in ratios of 10:90 [+:−] and the other with ratios of 90:10 (100% [−] camphene in both trials). For each trail, we applied one stem-section equivalent of the "galled" blend to a 2.5-cm square of filter paper (Qualitative No. 1, Whatman, Maidstone, UK), another paper square was treated with a similar dosage of the "ungalled" blend (with α- and β-pinene enantiomers in ratios of 50:50 and 100% [−] camphene), and a third square of paper was treated with hexane. We allowed solvent to evaporate and arranged the three paper squares in a $\sim$8-cm diam circle within a 10-cm diam Petri dish. Individual males were released at the dish center, and we recorded how long they remained on each paper square during a 5-min period, and their behaviors. Wasps were from the same source as above. We replicated the bioassay 20 times for each trial, replacing the paper squares and changing their position after every 5 wasps. We tested differences between treatments in the amount of time wasps spent on paper squares with ANOVA (SAS Institute, 2001). Differences between individual treatment means were tested with the LSD means separation test (Sokal and Rohlf, 1995).

RESULTS AND DISCUSSION

Response to Galled and Ungalled Stems. In the "Y"-configuration test, 34 of 38 male *A. rufus* responded to the galled stems ($X^2 = 23.7$, $P < 0.001$). On seven occasions, wasps walked onto the ungalled stem first, but immediately walked off and onto the galled stem where they remained. Moreover, wasps exhibited the characteristic searching behavior only on galled stems, while on ungalled

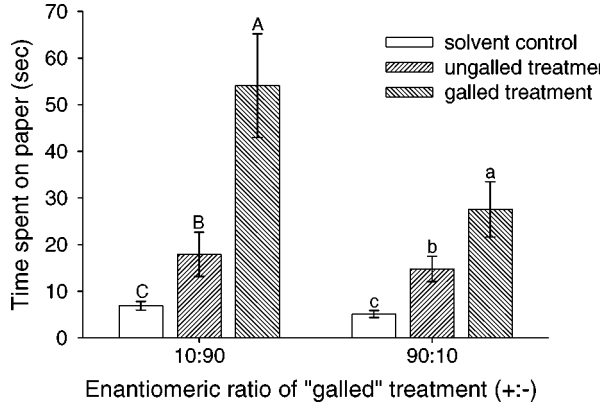

FIG. 1. Relationship between amount of time that male *Antistrophus rufus* spent on pieces of filter paper and ratios of enantiomers of α- and β-pinene with which the paper was treated. In one trial (group of bars on left), the pinene blend was representative of galled stems with a 10:90 [+:−] enantiomeric ratio of both compounds, while this ratio was skewed in the opposite direction [90:10] in the second trial (bars on right; including 100% [−] camphene in both trials; see text for explanation of treatments). Pinene blends representative of ungalled stems had enantiomeric ratios of 50:50 (also including [−] camphene). In both trials, male wasps spent significantly more time on paper representative of galled stems than on "ungalled" paper and solvent controls (Trial with pinenes in enantiomeric ratios of 10:90: ANOVA $F_{2,55} = 12.1$, $P < 0.001$; trial with 90:10: ANOVA $F_{2,59} = 8.8$, $P < 0.001$). Bars marked with different letters of the same case are significantly different (LSD $P < 0.05$).

stems they walked in a directed manner, infrequently antennating the stem surface. These findings are further evidence that male *A. rufus* use volatile plant cues to discriminate between stems that contained galls and those that do not.

Response to Synthetic Compounds. In bioassays that tested the response, males remained on filter paper treated with the "galled" blend of monoterpenes for three to eight times longer than on solvent controls, and two to three times longer than on paper treated with the "ungalled" blend (Figure 1). Males showed a statistically significant, although weaker response to the "ungalled" blend relative to the solvent control (Figure 1). Consistent with their behavior on plant stems in the "Y"-configuration test, males usually displayed their characteristic searching behavior on filter paper treated with the "galled" blend, but rarely did so on paper treated with the "ungalled" blend or the solvent control.

Our hypothesis that mate location by male *A. rufus* is cued by changes in monoterpene stereochemistry that are associated with galls of conspecifics is supported by their ability to discriminate between galled and ungalled plant stems, by chemical differences between the two types of stems, and by the corresponding response of males to blends of synthetic compounds that mimic volatile profiles

of plant stems. Monoterpene stereochemistry also influences the behavior of other insects (e.g., Erbilgin and Raffa, 2001); however, our studies are apparently the first to demonstrate that changes in monoterpene stereochemistry that are associated with the insect itself play a central role in location of mates.

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STRAIGHT AND BRANCHED-CHAIN FATTY ACIDS IN PREORBITAL GLANDS OF SIKA DEER, *Cervus nippon*

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Abstract—Using GC-MS analysis, 11 major volatile compounds were found in the preorbital gland secretion from a female sika deer, *Cervus nippon*. These compounds are the C_{14} through C_{18} straight-chain fatty acids, (*Z,Z*)-9,12-octadecadienoic acid, 12-methyltridecanoic acid, 13-methyltetradecanoic acid, 14-methylpentadecanoic acid, 14-methylhexadecanoic acid, and 15-methylhexadecanoic acid. The five branched-chain acids make up over 29% of the volatiles in the gland. This is the first time branched-chain carboxylic acids have been reported from ungulate preorbital glands.

Key Words—*Cervus nippon*, Cervidae, sika deer, preorbital glands, straight-chain fatty acids, branched-chain fatty acids.

INTRODUCTION

Secretion from the preorbital skin glands of many ungulate species has been shown to be used for scent marking. The components of preorbital gland secretion have been identified for many species of African antelope (Bovidae): suni, *Neotragus moschatus* (Stander et al., 2002); bontebok, *Damaliscus dorca dorcas* (Burger et al., 1999b); blesbok, *D. d. phillipsi* (Burger et al., 1999b); steenbok, *Raphicerus campestris* (Burger et al., 1999a); klipspringer, *Oreotragus oreotragus* (Burger et al., 1997); grysbok, *R. melanotis* (Burger et al., 1996); oribi, *Ourebia ourebi* (Mo et al., 1995); grey duiker, *Sylvicapra grimmia* (Burger et al., 1988); red duiker, *Cephalophus natalensis* (Burger et al., 1988); and blue duiker, *Cephalophus monticola* (Burger and Pretorius, 1987). The preorbital gland secretion from another bovid, the arctic muskox, *Ovibos moschatus* (Flood et al., 1989) has also been investigated.

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The composition of the preorbital gland secretion has been reported for only one member of the Cervidae, the reindeer, *Rangifer tarandus* (Andersson, 1979). This communication reports the major volatile components from the preorbital gland of another cervid, the sika deer, *Cervus nippon.* Sika are a medium sized deer that were originally found in Eastern Siberia, Japan, Manchuria, Formosa, and China. Numerous introductions of sika have been made throughout the world. In the United States, free ranging populations are found in Texas, Maryland, Wisconsin, and Virginia.

METHODS AND MATERIALS

The preorbital secretion from a single, free ranging, adult sika doe from Kerr County, Texas, USA, was collected during March 2003. The glandular secretions, obtained from both preorbital glands were combined and placed in a glass vial with a Teflon®-lined stopper containing 2.0 ml of CH_2Cl_2. A control sample, hair and attached skin lipids, was taken from in back of the neck and likewise preserved in CH_2Cl_2. A sample of the solvent was saved for analysis. Samples were carried at ambient temperature to Arcata, California, USA and analyzed by gas chromatography—mass spectrometry (GC-MS) within 3d of collection.

The GC-MS analyses were performed on the CH_2Cl_2 extracts in a splitless mode (0.5 min), using an Hewlett-Packard GCD Plus fitted with a 30-m $\times$ 0.25-mm cross-linked phenyl methyl silicone capillary column (HP-5MS). The gas chromatograph was programmed so the oven temperature was kept at 40°C for 4 min, then increased to a final temperature of 325°C at a rate of 30°C/min, and held at this temperature for 5 min. Mass spectral ions below $m/z = 39$ were not recorded. The relative amount of each component was determined as the percent of the total ion current (TIC). Impurities identified in the solvent and/or the control were not reported. Authentic samples were purchased from Sigma-Aldrich Chemical Co., Milwaukee, Wisconsin and Fisher Scientific (Arcos), Pittsburgh, Pennsylvania.

RESULTS AND DISCUSSION

GC-MS analysis of the preorbital gland secretion *Cervus nippon*, showed it contains 11 major components, all free fatty acids ranging from C_{14} to C_{18} (Table 1). Five branched-chain acids make up over 29% of the volatiles in the gland. This is the first time branched-chain carboxylic acids have been reported from ungulate preorbital glands. Initial identification of the C_{14} to C_{18} straight-chain fatty acids and (*Z,Z*)-9,12-octadecadienoic acid in the secretion was done by comparison of mass spectra in the NIST 1998 computerized mass spectral library. The structures of the branched-chain were deduced from their relative

TABLE 1. MAJOR VOLATILE COMPOUNDS IN THE PREORBITAL SECRETION OF SIKA DEER

Compound	Retention time (min)	% of TIC
12-Methyltridecanoic acid	10.41	2.1
Tetradecanoic acid	10.55	5.2
13-Methyltetradecanoic acid	10.79	12.9
Pentadecanoic acid	10.91	3.6
14-Methylpentadecanoic acid	11.13	9.9
Hexadecanoic acid	11.27	54.0
15-Methylhexadecanoic acid	11.45	1.7
14-Methylhexadecanoic acid	11.48	2.4
Heptadecanoic acid	11.57	1.0
(*Z*,*Z*)-9,12-Octadecadienoic acid	11.81	3.7
Octadecanoic acid	11.87	3.5

retention times and analysis of their mass spectra. Molecular ions were observed for all compounds. All identifications were confirmed by comparison of spectra and retention times to those of authentic standards.

The EI-MS (15 largest peaks) of the branched-chain carboxylic acids are: 12-methyltridecanoic acid, m/z = 185(34), 129(29), 87(17), 85(19), 83(22), 73(89), 71(31), 69(35), 60(82), 57(65), 56(20), 55(56), 43(100), 42(19), and 41(72); 13-methyltetradecanoic acid, m/z = 129(25), 87(17), 85(18), 83(20), 73(80), 71(30), 69(32), 61(17), 60(75), 57(63), 56(20), 55(54), 43(100), 42(18), and 41(69); 14-methylpentadecanoic acid, m/z = 129(24), 97(15), 85(18), 83(20), 73(77), 71(30), 69(32), 61(18), 60(73), 57(64), 56(20), 55(55), 43(100), 42(17), and 41(67); 14-methylhexadecanoic acid, m/z = 129(13), 97(21), 85(16), 83(26), 73(46), 71(32), 70(16), 69(39), 60(54), 57(97), 56(33), 55(75), 43(96), 42(19), and 41(100); and 15-methylhexadecanoic acid, m/z = 129(25), 97(15), 85(18), 83(20), 73(73), 71(32), 69(33), 61(19), 60(69), 57(67), 56(21), 55(55), 43(100), 42(16), and 41(63).

The preorbital secretion of sika differs from the composition reported for most other species as it contains relatively few compounds, all carboxylic acids. Only klipspringer preorbital glands contain a fewer number of volatile compounds, four ketones and four esters (Burger et al., 1997). Preorbital glands of reindeer, the only other member of the Cervidae for which this secretion has been studied, contain free and esterified cholesterol, lanosterol, fatty acids, triglycerides, and the ketones, 4-heptanone and 2-methyl-4-heptanone (Andersson, 1979). In African antelope, preorbital secretions usually contain many components with several different types of chemical groups. Some of the major compounds in the glands of these species include: formate esters in suni, grysbok, steenbok, and oribi (Burger et al., 1996, 1999a; Mo et al., 1995; Stander et al., 2002); ketones in bontebok, blesbok, klipspringer, and blue duiker (Burger et al., 1997, 1999b; Burger and

Pretorius, 1987); and thiazoles in grey and red duiker (Burger et al., 1988). Major components in the secretion of the arctic muskox are saturated lactones along with fatty acids, cholesterol and other compounds (Flood et al., 1989).

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IMPACT OF 10 DIETARY STEROLS ON GROWTH AND REPRODUCTION OF *Daphnia galeata*

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Abstract—In crustaceans, cholesterol is an essential nutrient, which they must directly obtain from their food or by bioconversion from other dietary sterols. Eukaryotic phytoplankton contain a great variety of sterols that differ from cholesterol in having additional substituents or different positions and/or number of double bonds in the side chain or in the sterol nucleus. In this study, we investigated to what extent these structural features affect the growth and reproduction of *Daphnia galeata* in standardized growth experiments with the cyanobacterium *Synechococcus elongatus* supplemented with single sterols as food source. The results indicated that Δ^5 (sitosterol, stigmasterol, desmosterol) and $\Delta^{5,7}$ (7-dehydrocholesterol, ergosterol) sterols meet the nutritional requirements of the daphnids, while the Δ^7 sterol lathosterol supports somatic growth and reproduction to a significantly lower extent than cholesterol. Dihydrocholesterol (Δ^0) and lanosterol (Δ^8) did not improve the growth of *D. galeata*, and growth was adversely affected by the Δ^4 sterol allocholesterol. Sterols seem to differ in their allocation to somatic growth and reproduction. Thus, structural differences of dietary sterols have pronounced effects on life-history traits of *D. galeata*.

Key Words—Food quality, cyanobacteria, dietary sterols, cholesterol, *Daphnia galeata.*

INTRODUCTION

The transfer of energy from primary producers to higher trophic levels is an important factor that determines the trophic structure of aquatic food webs. At the phytoplankton–zooplankton interface, the efficiency of carbon transfer is highly variable. This variation can be attributed to the changing nutritional value of phytoplankton assemblages. Nutritional inadequacy can be due to toxicity (Lampert,

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1981a,b), digestive resistance (Porter and McDonough, 1984), or mineral (Elser et al., 2001) or biochemical composition of phytoplankton species, and can result in a decoupling of primary and secondary production. The biochemical composition of phytoplankton, in particular the content of polyunsaturated fatty acids (PUFAs), has been discussed as being potentially limiting for *Daphnia* growth (Ahlgren et al., 1990; Müller-Navarra, 1995; Wacker and Von Elert, 2001).

PUFAs are of special importance for freshwater zooplankton nutrition in lakes dominated by cyanobacteria, as articulated in a correlative study by Müller-Navarra et al. (2000). Cyanobacteria in general lack long-chain PUFAs (Cobelas and Lechardo, 1988; Ahlgren et al., 1992), and the well-known low carbon-transfer efficiency at the cyanobacteria–*Daphnia* interface has been suggested to be caused by a deficiency in long-chain PUFAs (Müller-Navarra et al., 2000). Supplementation of the cyanobacterium *Synechococcus elongatus* with a PUFA-rich fish oil emulsion leads to better growth and reproduction of *Daphnia* (DeMott and Müller-Navarra, 1997) and, therefore, supports the correlative evidence. However, Von Elert and Wolffrom (2001), have found that the absence of a non-PUFA lipid present in eukaryotic algae constrains assimilation of cyanobacterial carbon. Fish oil contains other lipids in addition to PUFAs, such as sterols, which are also essential for growth and reproduction of crustaceans (Goad, 1981). Cyanobacteria, as prokaryotes, lack or contain only traces of sterols (Hai et al., 1996, Volkman, 2003). In a previous study, we have shown that the low carbon-transfer efficiency of cyanobacteria to *Daphnia galeata* is caused by the lack of sterols in cyanobacteria (Von Elert et al., 2003).

Like all arthropods, crustaceans are incapable of synthesizing sterols de novo and, therefore, must acquire these essential nutrients from their diet (Goad, 1981). Crustaceans generally have a simple sterol composition with characteristic high cholesterol levels (Teshima and Kanazawa, 1971a; Yasuda, 1973). Cholesterol is an indispensable structural component of cell membranes and serves as a precursor for many bioactive molecules, such as ecdysteroids, which are involved in the process of molting (Goad, 1981; Harrison, 1990). However, the herbivorous cladoceran *Daphnia*, unlike carnivorous crustaceans, cannot rely on a dietary source of cholesterol because only trace amounts are found in many phytoplankton species (Nes and McKean, 1977). Eukaryotic phytoplankton contain a great variety of plant sterols (Nes and McKean, 1977; Volkman, 2003), which can be distinguished from cholesterol by their chemical structure. These phytosterols are often characterized by additional substituents or by the position and/or number of double bonds in the side chain or in the sterol nucleus (Piironen et al., 2000). The crustaceans examined to date are capable of converting dietary sterols to cholesterol (Teshima, 1971; Teshima and Kanazawa, 1971b; Ikekawa, 1985; Harvey et al., 1987), but not all sterols are suitable precursors for the synthesis of cholesterol (Teshima et al., 1983).

Under field conditions, the diet of the nonselectively suspension-feeding *D. galeata* is complex. The diet usually consists of phytoplankton, protozoa, bacteria, and detritus in varying ratios. Depending on the composition of their diet, the cladocerans are provided with a large variety of sterols in different quantities. De Lange and Arts (1999) correlated biochemical variables of natural seston with *Daphnia* growth rates and found that the sterol content is a useful tool to predict *Daphnia* growth. However, growth of the herbivorous zooplankton might not only be limited by the total sterol content itself, but also by the absence of sterols that are suitable precursors of cholesterol. In periods when phytoplankton assemblages are dominated by species with an unsuitable sterol pattern, growth and reproduction of *Daphnia* could be constrained by the low availability of suitable sterols. The first evidence that structural differences of dietary sterols can have pronounced effects on life-history traits of arthropods has been found in terrestrial systems. Behmer and Grebenok (1998) pointed out that growth and fecundity of the moth *Plutella xylostella* was affected by dietary sterols. Further on, it was recently demonstrated that sterols with double bonds at Δ^7 and/or Δ^{22} (Figure 1) failed to support development of different grasshopper species and that survival of the grasshopper *Schistocerca americana* was constrained by the ratio of suitable to unsuitable sterols in their diet (Behmer and Elias, 2000). Consistently, the development of marine copepods was negatively affected by Δ^7 sterols, whereas Δ^5 sterols allowed a rapid development of the copepods (Klein Breteler et al., 1999). Comparable investigations on the structural requirements of freshwater zooplankton with regard to sterols are missing to date.

The aim of this study was to investigate to what extent structural features of sterols, such as the alkylation of the side chain or the presence or absence of double bonds, affect the nutritional value of single sterols for *Daphnia*. Standardized growth experiments of *D. galeata* with the cyanobacterium *S. elongatus* supplemented with single sterols as food source were conducted. *S. elongatus* is well assimilated by *Daphnia* (Lampert, 1977a,b) and does not contain any sterols. Thus, the cyanobacterium is a convenient source of carbon and a useful "transfer vehicle" for delivering sterols to the daphnids.

METHODS AND MATERIALS

Cultures and Growth Experiments. Laboratory growth experiments were conducted with a clone of *Daphnia galeata*, which was originally isolated from Lake Constance (Stich and Lampert, 1984). The green alga *Scenedesmus obliquus* (SAG 276-3a, Sammlung von Algenkulturen Göttingen, Germany) was grown in batch culture and harvested in the late-exponential phase. It was used as the food source for the stock culture of *D. galeata* and for the newborn experimental

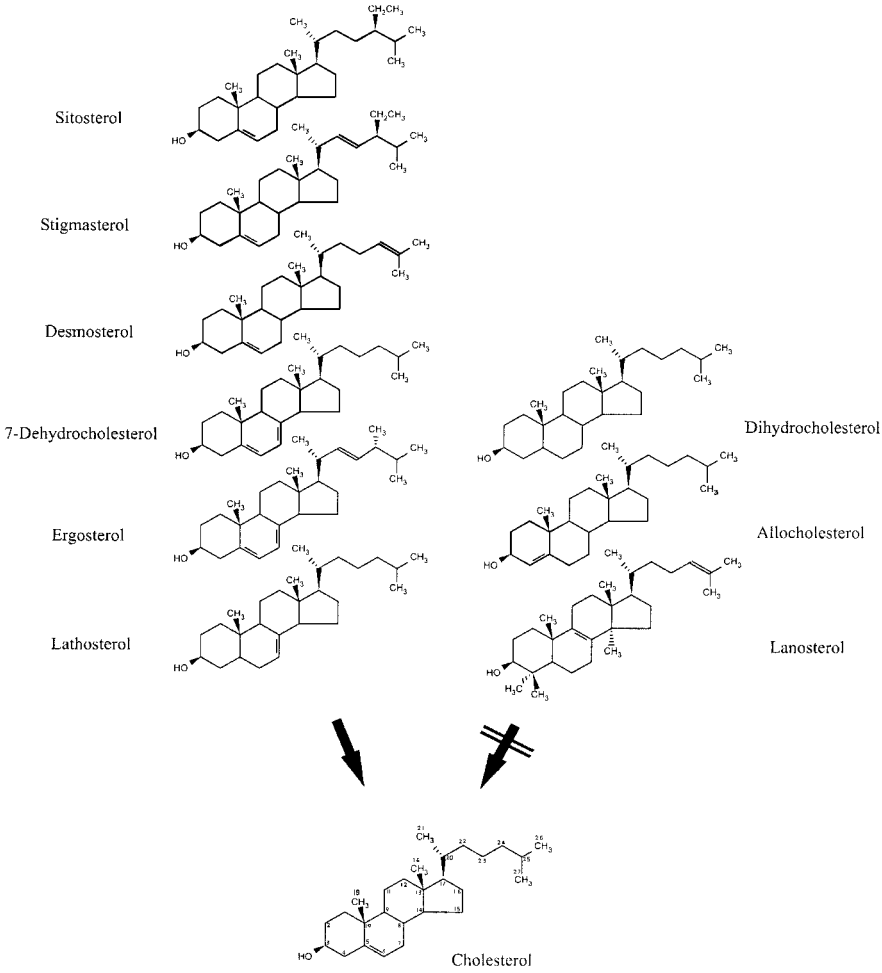

FIG. 1. Structural requirements for the conversion of dietary sterols to cholesterol in *Daphnia galeata*. Sterols on the left are suitable precursors for the synthesis of cholesterol, whereas sterols on the right are not. Potential intermediates in sterol metabolism are not shown.

animals, which were cultured to the age of 48 hr in a flow-through system prior to the growth experiments. *S. elongatus* (SAG 89.79) was grown in chemostats at a dilution rate of 0.25 d^{-1} according to Von Elert and Wolffrom (2001). *S. elongatus* and *S. obliquus* were grown in Cyano medium (Jüttner et al., 1983). Chemostat-grown cells were concentrated by centrifugation and resuspended in fresh medium. Carbon concentrations of the cyanobacterial suspensions were

estimated from photometric light extinction (800 nm) using carbon-extinction equations. *S. elongatus* had a molar C:N:P ratio of 121:23:1. Growth experiments were carried out at 20°C in glass beakers filled with 0.5 l of filtered lake water (0.45 μm pore-sized membrane filter) containing 2 mg C l^{-1} *S. elongatus*. The 48-hr-old juveniles (released from the third clutch within 10 hr) were transferred from the flow-through system into these beakers. The food suspensions were renewed daily within the 4 d of the experimental period. Somatic growth rates (g) were determined as the increase in dry weight (W) during the experiments using the equation:

$$g = \frac{\ln W_t - \ln W_0}{t}.$$

Subsamples of the experimental animals were taken at the beginning (W_0) and at the end (W_t) of an experiment. The subsamples consisting of ~15 juveniles were dried for 12 hr and weighed on an electronic balance (Mettler UMT 2; ± 0.1 μg). Each treatment consisted of three replicates with 15 animals each, and growth rates were calculated as means for each treatment.

Supplementation of Sterols. Sterols used for supplementation are given in Table 1, they were selected according to their chemical structure and their natural occurrence. To enrich *S. elongatus* with sterols, 10 mg bovine serum albumin (BSA) was dissolved in 5 ml of ultra-pure water, and 200 μl of an ethanolic stock solution of the free sterol (2.5 mg ml^{-1}) were added. Subsequently, 4 mg particulate organic carbon (POC) of the *S. elongatus* stock solution were added to each solution, and the volume was brought to 40 ml with Cyano medium. The resulting suspension was incubated on a rotary shaker (100 revolutions min^{-1}) for 4 hr. Surplus BSA and free sterols were removed by washing the cells three times in 10 ml fresh medium according to Von Elert (2002). The resulting *S. elongatus* suspension was used as food in the growth experiments.

TABLE 1. NOMENCLATURE OF STEROLS SUPPLEMENTED TO THE *Daphnia galeata* FOOD SOURCE, *Synechococcus elongatus*

Trivial name	IUPAC name	Formula	Commercial source
Cholesterol	Cholest-5-en-3β-ol	$C_{27}H_{46}O$	Sigma C-8667
Stigmasterol	Stigmasta-5,22-dien-3β-ol	$C_{29}H_{48}O$	Sigma S-2424
Sitosterol	Stigmast-5-en-3β-ol	$C_{29}H_{50}O$	Sigma S-1270
Ergosterol	(22E)-Ergosta-5,7,22-trien-3β-ol	$C_{28}H_{44}O$	Sigma E-6510
Lathosterol	5α-Cholest-7-en-3β-ol	$C_{27}H_{46}O$	Sigma C-3652
Dihydrocholesterol	5α-Cholestan-3β-ol	$C_{27}H_{48}O$	Sigma D-6128
Lanosterol	5α-Lanosta-8,24-en-3β-ol	$C_{30}H_{50}O$	Sigma L-1504
Allocholesterol	Cholest-4-en-3β-ol	$C_{27}H_{46}O$	Steraloids C6100
7-Dehydrocholesterol	Cholesta-5,7-dien-3β-ol	$C_{27}H_{44}O$	Steraloids C3000
Desmosterol	Cholesta-5,24-dien-3β-ol	$C_{27}H_{44}O$	Steraloids C3150

Analyses. Sterols were analyzed from approximately 0.5 mg POC of the food suspensions filtered on precombusted GF/F filters or from 60 to 80 animals washed twice with ultra-pure water. Lipids were extracted three times with dichloromethane:methanol (2:1 (v/v)). After saponification with 0.2 mol l^{-1} methanolic KOH (70°C, 1 hr) and addition of ultra-pure water, the neutral lipids (sterols) were partitioned into *iso*-hexane:diethyl ether (9:1 (v/v)). The sterols were analyzed as free sterols with a gas chromatograph (HP 6890) equipped with an HP-5 capillary column (Agilent) and a flame ionization detector. The carrier gas (helium; purity 5.0) had a flow rate of 1.5 ml min^{-1}. The temperature was raised from 150 to 280°C at 15°C min^{-1} and increased to 330°C at 2°C min^{-1}. The final temperature was held for 5 min. Sterols were quantified by comparison to 5α-cholestan, which was used as an internal standard and identified using a gas chromatograph–mass spectrometer (Finnigan MAT GCQ) equipped with a fused silica capillary column (DB-5MS, J&W). Spectra were recorded between 60 and 400 amu in the EI ionization mode. POC was determined with an NCS-2500 analyzer (Carlo Erba Instruments).

Data Analysis. All data were analyzed using one-way analysis of variance (ANOVA). For growth rates and clutch sizes, raw data met the assumption of homogeneity of variance; values of the supplemented sterols and cholesterol in *D. galeata* were $\log_{10}$-transformed to meet assumptions for ANOVA. The effects of single treatments were tested by Tukey's HSD post hoc tests. A significance level of $P = 0.05$ was applied to all statistical analyses.

RESULTS

Growth Experiments. Growth of *D. galeata* on unsupplemented *S. elongatus* was in general poor (growth rate, g = 0.07 d^{-1}). Supplementation of *S. elongatus* with sterols affected somatic growth of *D. galeata* (ANOVA, $F_{10,22} = 389$; $P < 0.001$; Figure 2). Growth rates on cyanobacteria supplemented with stigmasterol ($g = 0.30$ d^{-1}), sitosterol ($g = 0.32$ d^{-1}), ergosterol ($g = 0.32$ d^{-1}), and 7-dehydrocholesterol ($g = 0.30$ d^{-1}) were highest and significantly different from growth rates with the other treatments (Tukey's HSD, $P < 0.05$). Supplementation with desmosterol also led to a high growth rate ($g = 0.28$ d^{-1}), but was significantly lower than the growth rates obtained with sitosterol, ergosterol, and 7-dehydrocholesterol. Supplementation with cholesterol had a less-pronounced effect on growth ($g = 0.24$ d^{-1}) than supplementation with the sterols mentioned above. Dihydrocholesterol ($g = 0.09$ d^{-1}) and lanosterol ($g = 0.08$ d^{-1}) did not improve growth, compared to growth of animals reared on unsupplemented *S. elongatus* (dihydrocholesterol, $P = 0.92$; lanosterol, $P = 1$). Negative growth rates were observed after supplementation with allocholesterol ($g = -0.03$ d^{-1}).

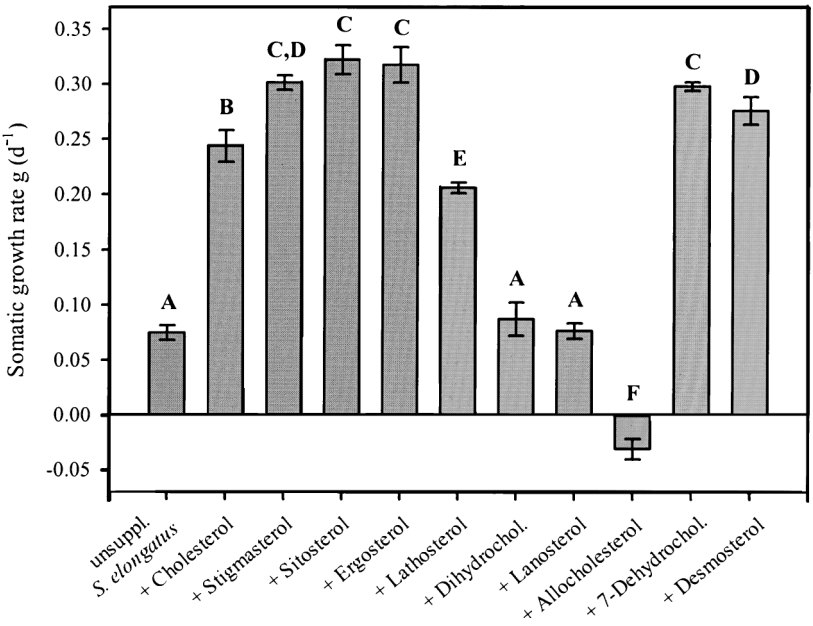

FIG. 2. Somatic growth of *Daphnia galeata* reared on *Synechococcus elongatus* unsupplemented and supplemented with single sterols. Data are means of three replicates per treatment; error bars indicate SD. Bars labeled with the same letters are not significantly different (Tukey's HSD, $P < 0.05$ following ANOVA).

Clutch sizes exhibited almost the same pattern as the growth rates (Figure 3). However, supplementation with 7-dehydrocholesterol led to the highest clutch size of 2.4 eggs per individual, whereas the growth rate obtained with 7-dehydrocholesterol did not differ from those obtained after supplementation with stigmasterol, sitosterol, and ergosterol. *D. galeata* fed on *S. elongatus* supplemented with stigmasterol produced 1.5 eggs per individual, which is significantly less than animals fed *S. elongatus* supplemented with sitosterol and ergosterol (Tukey's HSD, $P < 0.05$ following ANOVA, $F_{7,16} = 106$; $P < 0.001$). Although dihydrocholesterol did not improve growth, *D. galeata* did produce eggs in this treatment, with a clutch size of 0.2 eggs per individual. Animals kept on a diet supplemented with lanosterol or allocholesterol and animals fed pure *S. elongatus* did not produce eggs within the 4-d experiment.

Sterol Analysis. No sterols other than the supplemented sterols were detected in *S. elongatus*, which indicated that the supplemented sterols were not metabolically converted in the cyanobacterium. After feeding *D. galeata* 4 d on supplemented *S. elongatus*, all supplemented sterols could be detected in the animals (Figure 4), but the amounts per individual differed (ANOVA, $F_{9,20} = 45.5$;

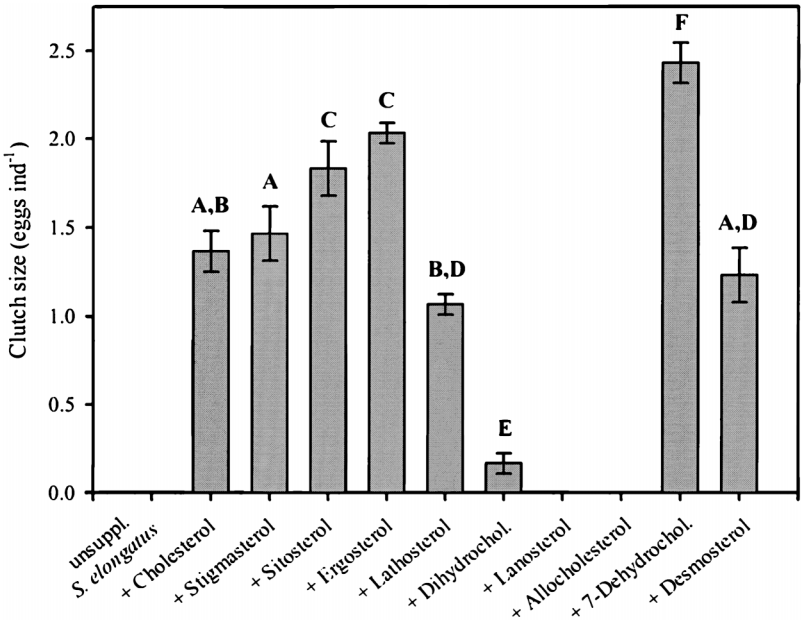

FIG. 3. Number of eggs of the first clutch of *Daphnia galeata* feeding on *Synechococcus elongatus* unsupplemented and supplemented with single sterols. Data are means of three replicates per treatment; error bars indicate SD. Bars labeled with the same letters are not significantly different (Tukey's HSD, $P < 0.05$ following ANOVA).

$P < 0.001$). Cholesterol was the main sterol found in *D. galeata* in all experimental treatments. *D. galeata* fed on *S. elongatus* supplemented with cholesterol had a higher cholesterol content than animals grown on unsupplemented *S. elongatus* (Figure 4). The amounts of supplemented sitosterol, dihydrocholesterol, lanosterol, and 7-dehydrocholesterol in *D. galeata* were higher than the amounts of supplemented stigmasterol, ergosterol, and allocholesterol in the animals. Only small amounts of lathosterol and desmosterol were detected in *D. galeata* reared on food supplemented with these sterols.

Immediately prior to the experiments, newborn animals were raised for 2 d on the green alga *Scenedesmus obliquus*. In addition to cholesterol, small amounts of the three major phytosterols of *S. obliquus* (Von Elert et al., 2003) were detected in these animals. Although no cholesterol was found in *S. obliquus*, the cholesterol content of *D. galeata* increased after growth for an additional 4 d on the green alga (Table 2), which indicated that the phytosterols present in *S. obliquus* were converted to cholesterol. In contrast, the cholesterol content of *D. galeata* decreased when the 2 d-old animals were fed an additional 4 d on the

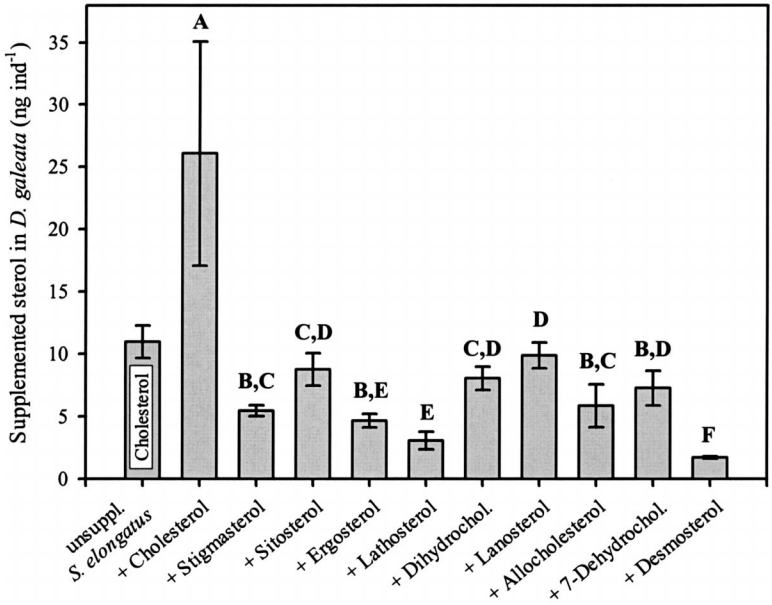

FIG. 4. Sterol content in *Daphnia galeata*, grown on *Synechococcus elongatus* unsupplemented and supplemented with single sterols. At the end of the experiment animals were analyzed for the content of the supplemented sterol. For animals grown on unsupplemented *S. elongatus*, the cholesterol content is given. Data are means of three replicates per treatment; error bars indicate SD. Bars labeled with the same letters are not significantly different (Tukey's HSD, $P < 0.05$ following ANOVA, $F_{9,20} = 45.5$; $P < 0.001$).

unsupplemented cyanobacterium *S. elongatus* (Table 2). With the assumption that cholesterol in *D. galeata* arises from the conversion of dietary sterols, the sterol-free cyanobacterium *S. elongatus* was supplemented with single sterols, and the effect of the supplemented sterols on the cholesterol content of *D. galeata* was

TABLE 2. CHOLESTEROL CONTENT OF *Daphnia galeata* AT THE AGE OF 2 AND 6 DAYS

Food regime	Cholesterol content (ng ind^{-1}) $\pm$ SD
2 days on *Scenedesmus obliquus*	22.55 ± 0.57
6 days on *Scenedesmus obliquus*	54.52 ± 9.44
2 days on *Scenedesmus obliquus*/ 4 days on *Synechococcus elongatus*	10.96 ± 1.30

[a] The animals either were reared continuously on *Scenedesmus obliquus* or were fed with *Synechococcus elongatus* after the second day.

All cholesterol contents were significantly different (Tukey's HSD following ANOVA $F_{2,6} = 132$; $P < 0.001$). Means values of three replicates per treatment are given.

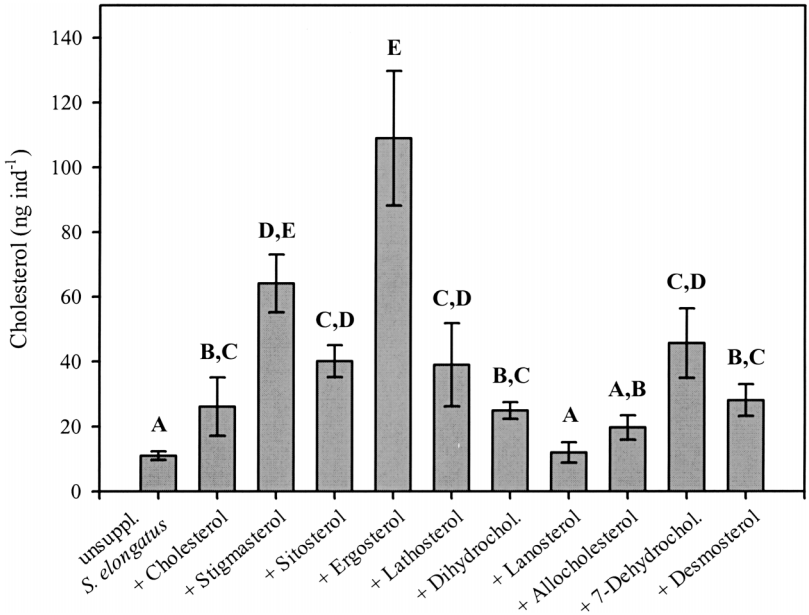

FIG. 5. Cholesterol content of *Daphnia galeata* reared on *Synechococcus elongatus* unsupplemented and supplemented with single sterols. Data are means of three replicates per treatment; error bars indicate SD. Bars labeled with the same letters are not significantly different (Tukey's HSD, $P < 0.05$ following ANOVA, $F_{10,22} = 30.6$; $P < 0.001$).

examined (Figure 5). Animals fed *S. elongatus* supplemented with ergosterol had a tenfold higher cholesterol content (109 ng ind^{-1}) than animals grown on unsupplemented *S. elongatus* (11 ng ind^{-1}). Supplementation of cyanobacteria with ergosterol or stigmasterol led to a higher content of cholesterol in the daphnids than supplementation with cholesterol itself. Supplementation of the cyanobacterial food with sitosterol, lathosterol, dihydrocholesterol, 7-dehydrocholesterol, or desmosterol also increased the cholesterol content of *D. galeata*, which indicated that these sterols were also converted to cholesterol. Supplementation with lanosterol and allocholesterol, on the other hand, did not affect the cholesterol content of the daphnids, which suggested that neither of these sterols could be used as a cholesterol precursor by the animals.

DISCUSSION

The crustaceans examined to date are incapable of synthesizing sterols de novo—they require a dietary source of sterols to meet their basic physiological

demands. In a recently published study, we have shown that poor somatic growth of *Daphnia galeata* on *Synechococcus elongatus* is due to the lack of sterols in the cyanobacterium (Von Elert et al., 2003). Supplementation of *S. elongatus* with cholesterol improved the growth of the animals, which indicates that growth of *D. galeata* was limited by cholesterol. Since herbivorous crustaceans do not find sufficient amounts of cholesterol in their diet, they need to assimilate available dietary sterols and convert them to cholesterol (Ikekawa, 1985). Eukaryotic phytoplankton usually contain a variety of sterols that can be distinguished from cholesterol by their chemical structure. These sterols are often characterized by additional substituents or by the position and/or number of double bonds in the side chain or in the sterol nucleus (Piironen et al., 2000).

All supplemented sterols were detected in *D. galeata,* which indicates that they were assimilated by the animals. Although single sterols were found in relatively small amounts in *D. galeata,* the amounts were too high to be exclusively derived from ingested *S. elongatus* in the gut of the animals. In cases in which only small amounts of a single supplemented sterol were found in *D. galeata,* an increased cholesterol content of the animals was observed. In contrast, when a supplemented sterol was found in higher amounts in the animals, the cholesterol content was not affected. These two patterns provide evidence for which of the supplemented sterols can be converted to cholesterol by *D. galeata*.

In animals that are capable of synthesizing cholesterol de novo, the cyclization of squalene leads to lanosterol. Lanosterol differs from cholesterol by having additional C-4 dimethyl and C-14 methyl substituents and by the location of the double bond (Δ^8) in the sterol nucleus (Figure 1). Supplementation of cyanobacteria with lanosterol did not affect growth rates and clutch sizes of *D. galeata*. Furthermore, no increase in the cholesterol content of the animals was observed. The biochemical conversion of lanosterol to cholesterol involves the loss of the methyl groups, the removal of the Δ^8 double bond, and the introduction of a double bond at Δ^5. The above findings demonstrate that *D. galeata* lacks the enzymatic ability to convert Δ^8 sterols to cholesterol. Notwithstanding our findings, the conversion of Δ^8 sterols to cholesterol was hypothesized by Harvey et al. (1987) in the marine copepod *Calanus*. This suggests taxon specific differences in the structural requirements of dietary sterols for crustaceans.

The phytosterols sitosterol and stigmasterol differ from cholesterol in having an ethyl group at C-24, and stigmasterol has an additional double bond at Δ^{22} in the side chain (Figure 1). Sitosterol and stigmasterol are commonly found in higher plants and are also present in a number of microalgae (Volkman, 2003). The synthesis of cholesterol from these sterols requires a dealkylation at C-24. An efficient phytosterol C-24-dealkylating system is found in various crustacea (Ikekawa, 1985). Teshima (1971) has described the bioconversion of sitosterol to cholesterol in the prawn *Penaeus japonicus* using ^{14}C-labeled sitosterol. Our findings that food supplemented with sitosterol or stigmasterol led to an increased

cholesterol content of the animals indicates that a 24-dealkylation also occurs in *D. galeata*. Furthermore, *D. galeata* seems to be capable of saturating the additional Δ^{22} bond of stigmasterol during its transformation to cholesterol. However, sitosterol and stigmasterol improved growth more efficiently than cholesterol, which might indicate that *D. galeata* is also able to use these sterols directly without the circuitous synthesis of cholesterol and that these sterols play a yet unknown role in the metabolism of *D. galeata*.

Supplementation of cyanobacteria with desmosterol stimulated growth and egg production of *D. galeata* and increased the cholesterol content of the animals, which demonstrates that the ability to transform desmosterol to cholesterol is also present in *D. galeata*. The $\Delta^{5,24}$ diene desmosterol (Figure 1) is the terminal intermediate in the conversion of plant sterols (e.g., sitosterol and stigmasterol) to cholesterol in insects (Svoboda and Thompson, 1985). A Δ^{24} sterol reductase that reduces the double bond in the side chain, thereby converting desmosterol to cholesterol, has been found in the tobacco hornworm, *Manduca sexta* (Svoboda and Thompson, 1985). Experiments with labeled sterols have shown that the prawn *P. japonicus* also possesses the ability to use desmosterol as a precursor for the synthesis of cholesterol (Teshima and Kanazawa, 1973).

D. galeata is able to convert $\Delta^{5,7}$ sterols to Δ^{5} sterols, as evidenced by the large increase in the cholesterol content of the animals after supplementation of the food with 7-dehydrocholesterol ($\Delta^{5,7}$). 7-Dehyrocholesterol is found in the hemolymph and in particular in Y-organs of crustaceans, where molting hormones are synthesized (Lachaise et al., 1989; Rudolph et al., 1992). In many insects, 7-dehydrocholesterol is an intermediate in the transformation of cholesterol to ecdysteroids (Rees, 1985). Several studies suggest that 7-dehydrocholesterol is formed irreversibly from cholesterol in isolated prothoracic glands (Grieneisen, 1994). Here, we showed that a transformation of 7-dehydrocholesterol to cholesterol occurs in the cladoceran *D. galeata*. Assuming that cholesterol is the key sterol in crustaceans, it is surprising that 7-dehydrocholesterol improved the growth of the daphnids more efficiently than cholesterol. Synthesis of ecdysteroids from cholesterol requires the introduction of a Δ^{7} bond into the sterol nucleus, which is not necessary in the direct conversion of 7-dehydrocholesterol to ecdysteroids. The conversion of labeled 7-dehydrocholesterol to labeled ecdysteroids has been demonstrated by injection experiments with various insect species (see Grieneisen, 1994) and by incubation of fractionated Y-organs of the crab *Menippe mercenaria* with the sterol (Rudolph and Spaziani, 1992). Presumably, *D. galeata* is also capable of utilizing 7-dehydrocholesterol as a direct precursor of ecdysteroids. Increased clutch sizes relative to the growth rates showed that 7-dehydrocholesterol effectively supported egg production. Although it is generally assumed that effects of food quantity on somatic growth and on reproduction are highly correlated in juvenile *Daphnia* (Lampert and Trubetskova, 1996), it has been suggested that limitation by food quality might affect somatic growth and reproduction differently.

This has been shown for mineral (Urabe and Sterner, 2001) and biochemical (Becker and Boersma, 2003) aspects of food limitation. In accordance with these findings, sterols seem to differ in their allocation to somatic growth or reproduction. Further detailed investigations of sterol effects on life history are needed to reveal how these differences in allocation lead to differences in effects on fitness.

Supplementation of cyanobacteria with ergosterol resulted in a tenfold higher cholesterol content of *D. galeata* than in animals fed unsupplemented food. *D. galeata* is, therefore, capable of converting dietary ergosterol to cholesterol. Ergosterol, a $\Delta^{5,7,22}$ sterol, is found in most fungi, yeast, and in some species of green algae (Nes and McKean, 1977; Akihisa et al., 1992; Petkov and Kim, 1999). Ergosterol differs from 7-dehydrocholesterol in having an additional double bond at Δ^{22} in the side chain (Figure 1). Growth rates on food supplemented with ergosterol were as high as the growth rates reached with 7-dehydrocholesterol, sitosterol, and stigmasterol. The conversion of ergosterol to cholesterol requires the saturation at Δ^7 in the sterol nucleus, as described for 7-dehydrocholesterol, as well as saturation at Δ^{22} in the side chain, as described for stigmasterol. Only small amounts of ergosterol were detected in animals reared on ergosterol-supplemented food, which points to high metabolic transformation rates. Teshima and Kanazawa (1971b) have described the bioconversion of ergosterol to cholesterol in *Artemia salina* fed on ^{14}C-labeled *Euglena gracilis*. The ability to saturate the Δ^5 bond of a $\Delta^{5,7}$ diene, as discussed for 7-dehydrocholesterol, might also enable the direct conversion of ergosterol to ecdysteroids.

Supplementation with dihydrocholesterol, a completely saturated molecule (Δ^0), did not affect somatic growth of *D. galeata*, as compared with unsupplemented cyanobacteria, which indicates that a double bond in ring B is required for the conversion of dietary sterols to cholesterol (Figure 1). In contrast to somatic growth, egg production of the daphnids was positively affected by supplementation with dihydrocholesterol, which indicated the potential significance of sterols for reproduction. We are aware of only one example of the oxidation of a Δ^0 sterol to a Δ^5 sterol in arthropods: the firebrat, *Thermobia domestica*, is capable of synthesizing cholesterol from dihydrocholesterol (Svoboda and Thompson, 1985). Harvey et al. (1987) documented that ring-saturated stanols are poorly assimilated and that they pass unaltered through the gut of the marine copepod *Calanus*. In this study, we found significant amounts of the supplemented dihydrocholesterol in daphnid tissues, which indicates the assimilation of this stanol.

Allocholesterol and lathosterol differ from cholesterol in the position of the double bond in the sterol nucleus (Figure 1). Somatic growth of *D. galeata* was negatively affected by the supplementation with allocholesterol. A relocation of a double bond from Δ^4 to Δ^5, as required for the conversion of allocholesterol to cholesterol, seems improbable; however, we cannot exclude that a toxic effect of allocholesterol masked the enzymatic abilities of the animals. Supplementation with lathosterol increased the cholesterol content of the animals, which indicated

that lathosterol was converted to cholesterol. The conversion of lathosterol to cholesterol requires a shift of a double bond from Δ^7 to Δ^5, possibly via a $\Delta^{5,7}$ intermediate, as work with mammals has shown (Nes and McKean, 1977). Prahl et al. (1984) found that, compared with Δ^5 and $\Delta^{5,7}$ sterols, Δ^7 sterols were not readily removed during passage through the gut of the copepod *Calanus*. They speculated that dietary Δ^7 sterols can be used as precursors of ecdysteroids and that the poor assimilation of these sterols provides a mechanism to avoid a haphazard production of molting hormones. Alternatively, Prahl et al. (1984) suggested that *Calanus* simply lacks the ability to convert Δ^7 to $\Delta^{5,7}$ sterols and, therefore, the Δ^7 components are only poorly assimilated. The results of this study indicate that the Δ^7 sterol lathosterol was assimilated by *D. galeata* and converted to cholesterol. However, the observed growth rates were lower than those reached with food supplemented with cholesterol. The step Δ^7 to $\Delta^{5,7}$ involves the introduction of a double bond at Δ^5, which might be costly in terms of energy and, therefore, might be responsible for the lower growth rates as compared to those reached with supplementation with cholesterol.

Although this study shows that certain dietary sterols improve the somatic growth of *D. galeata*, there must be other factors that become limiting for the growth and reproduction of the herbivore, when the animals are released from sterol limitation. The maximal growth rates on sterol-supplemented *Synechococcus* ($g = 0.32\ \mathrm{d}^{-1}$) were below the almost maximal possible growth rate ($g = 0.5\ \mathrm{d}^{-1}$) of *D. galeata* fed on the green alga *Scenedesmus obliquus* (Wacker and Von Elert, 2001). Von Elert et al. (2003) already showed that the growth on cholesterol-supplemented *Synechococcus* was further improved by additional supplementation with PUFAs. Beside sterols and PUFAs there might be additional factors that determine the nutritional value of this coccal cyanobacterium to a lower extent.

Results derived from laboratory experiments are indispensable for determining the requirements of zooplankton species for single biochemical compounds, such as sterols, and provide a first step toward assessing the ecological relevance of these compounds under field conditions. During cyanobacterial blooms, the sterol content of the food will be low since only traces of sterols are found in prokaryotes (Hai et al., 1996; Volkman, 2003). This is corroborated by the observation that the total lipid levels (with sterols as a dominant lipid class) of *Daphnia pulex* from a hypereutrophic lake are at their lowest concentration during the height of the yearly *Aphanizomenon flos-aquae* bloom (Arts et al., 1992). In a previous laboratory study, we have shown that the absence of sterols constrains the carbon transfer between cyanobacteria and *D. galeata* (Von Elert et al., 2003). Compared to cyanobacteria, which do not provide sterols in sufficient amounts, eukaryotic phytoplankton contain a large variety of sterols (Nes and McKean, 1977; Volkman, 2003). However, specific phytoplankton classes or even single species could still be deficient in sterols suitable for supporting zooplankton growth.

If such species dominate the phytoplankton, sterol limitation of growth of *Daphnia* is possible. Thus, high levels of unsuitable sterols could adversely affect growth and reproduction of *Daphnia*, and can, therefore, be responsible for reduced fecundity and, projected at the population level, for reduced population growth.

In the field, sterols of phytoplankton can be subjected to transformation prior to their ingestion by the herbivorous crustaceans. Klein Breteler et al. (1999) have suggested that the poor quality of the chlorophycean *Dunaliella* for the development of marine copepods is due to a sterol deficiency of the alga. Furthermore, they have demonstrated that the chlorophycean food is biochemically upgraded by the heterotrophic dinoflagellate *Oxyrrhis marina* to high-quality copepod food. This trophic upgrading of food quality by an intermediary protozoan is attributed to sterol production in the dinoflagellate. The Δ^7 sterols present in *Dunaliella* do not support development of the copepods, whereas a rapid development of the copepods to the adult stage is observed when fed on *Oxyrrhis marina*, which contains primarily Δ^5 sterols. This example shows that unsuitable sterols in eukaryotic algae can constrain the development of herbivorous crustaceans. Intermediary grazers, such as protozoa, might biochemically upgrade such unsuitable phytoplankton species by adding more suitable sterols to the dietary carbon, thus determining the transfer efficiency of carbon from the microbial loop to metazoan grazers in natural systems.

In summary, this study provides evidence that sterols are essential dietary compounds that significantly affect growth and reproduction of *D. galeata*. Furthermore, the results showed that *D. galeata* is capable of converting dietary sterols to cholesterol, depending on their chemical structure. Particularly, Δ^5 and $\Delta^{5,7}$ sterols met the nutritional requirements of the animals, while the Δ^7 sterol lathosterol supported growth to a significantly lower extent than cholesterol. Dihydrocholesterol (Δ^0) and lanosterol (Δ^8) did not improve the growth of *D. galeata*, and growth was adversely affected by the Δ^4 sterol allocholesterol. Hence, structural features, particularly the configuration of the sterol nucleus, determine the nutritional value of dietary sterols. In insects, the pattern of sterol metabolism is by no means ubiquitous, and the nutritional dependency on specific sterols described for *D. galeata* might not be valid for crustaceans in general. In order to assess the ecological significance of certain sterols as potentially limiting biochemical resources, further detailed studies are required to reveal pathways and potential intermediates of sterol synthesis with regard to the nutritional requirements of freshwater zooplankton species. Von Elert et al. (2003) have already suggested that sterols could play a key role in determining carbon transfer efficiency from primary producers to herbivorous zooplankton. Here, we suggest that, in addition to low dietary sterol levels, the quality of dietary sterols could strongly affect the assimilation of dietary carbon.

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SPATIAL ARRANGEMENT OF ODOR SOURCES MODIFIES THE TEMPORAL ASPECTS OF CRAYFISH SEARCH STRATEGIES

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Abstract—In natural habitats, animals encounter cues from multiple odor sources that may impact foraging decisions. Previous work has focused on orientation behavior to one food odor source, and does not distinguish between mechanosensory and chemosensory guidance of orientation. The present study investigated how the spatial distribution of two food cues affects crayfish orientation behavior. Crayfish, *Orconectes virilis*, were presented with odor sources that were separated in an artificial stream. Orientation behavior was filmed from above and digitized at a rate of 1 frame per sec. Electrochemical recordings were taken to characterize the odor plume, and an acoustic doppler velocimeter was used to characterize the hydrodynamic structure of the artificial stream. Temporal changes in odor plume structure were seen as a result of the positioning of the odor sources. Changes in the intermittency of the odor pulses as well as concentration, rise time, and slope of the pulse were observed. Time series analysis showed that the lower frequency signals of the dual odor source were higher in energy than the single 1X or single 2X sources. Crayfish altered orientation strategies when presented with different spatial arrangements of food cues. In particular, the temporal aspects of the orientation pattern were most altered by the differences in odor presentations. Crayfish responded with faster walking speeds as a function of distance to the odor source and exhibited more consistent turning angles. This correlates with temporal changes in odor structure, and indicates that the temporal pattern of odor stimulation may be driving the temporal pattern of behavior.

Key Words—Orientation strategies, *Orconectes virilis*, hydrodynamics, odor plumes structure, temporal and spatial search strategies.

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INTRODUCTION

Crayfish must distinguish between complex odor signals and respond appropriately to locate mates, find food and prey, avoid predators, and select a habitat (Bouwma and Hazlett, 2001; Keller et al., 2001; Tomba et al., 2001). Here, we define complexity as an increase in range of temporal or spatial fluctuations within an odor plume. This complexity challenges the abilities of organisms to extract the necessary information to locate the source of an odor signal (Hazlett, 1999; Keller et al., 2001; Tomba et al., 2001).

In aquatic systems, various strategies are employed to orient toward an odor source. Blue crabs are thought to use an odor-gated rheotaxis to orient toward an odor source, i.e., they use the flow of water as spatial information and the concentration differences within and on the edges of the plume as temporal information to guide their movements (Weissburg and Zimmer-Faust, 1993; Zimmer-Faust et al., 1995; Finelli et al., 2000). Lobsters seem to rely more on a chemotactic strategy, i.e., they use both the temporal and spatial components of an odor plume to orient (McLeese, 1973; Moore et al., 1991; Atema, 1996). Crayfish appear to use a chemotactic strategy that is guided by spatial information within the odor plume (Moore and Grills, 1999; Keller et al., 2001; Tomba et al., 2001; Kraus-Epley and Moore, 2002). Therefore, the spatial or temporal distribution of the odor signal is critical for orientation behavior.

The fine-scale structure of chemical signals is important for its perception by organisms in their environment (Mafra-Neto and Cardé, 1995; Finelli et al., 1999; Moore et al., 2000). Three major factors could influence the spatial and temporal distribution of chemical signals: 1) the rate of release of a signal (Zimmer et al., 1999), 2) the hydrodynamic characteristics of the environment (Westerberg, 1991), and 3) the spatial location of odor sources in the habitat (Westerberg, 1991; Keller et al., 2001). Changes in odor plume structure have a strong effect on the efficiency of organisms to orient toward an odor source (Weissburg and Zimmer-Faust, 1993; Moore and Grills, 1999). For example, increases in turbulence as well as changes in pulse rate of the chemical signal altered plume variables, such as concentration within the patches and intermittency between the patches (Westerberg, 1991; Moore et al., 1994; Zimmer et al., 1999; Finelli et al., 2000; Moore et al., 2000).

Orientation behavior in crayfish becomes more efficient with increase of complexity of the odor signal (Moore and Grills, 1999; Keller et al., 2001; Tomba et al., 2001). Crayfish walk faster and spend more time moving toward the odor source with a wider range of temporal fluctuations in odor plumes. Changes in the physical habitat, i.e., substrate of the stream, alter the complexity of odor signals (Moore et al., 2000), and the spatial distribution of sources can alter orientation behavior of crayfish (Keller et al., 2001). The present study attempts to elucidate how changes in spatial–temporal aspects of an odor signal due to odor source placement alter the spatial–temporal orientation behavior of crayfish.

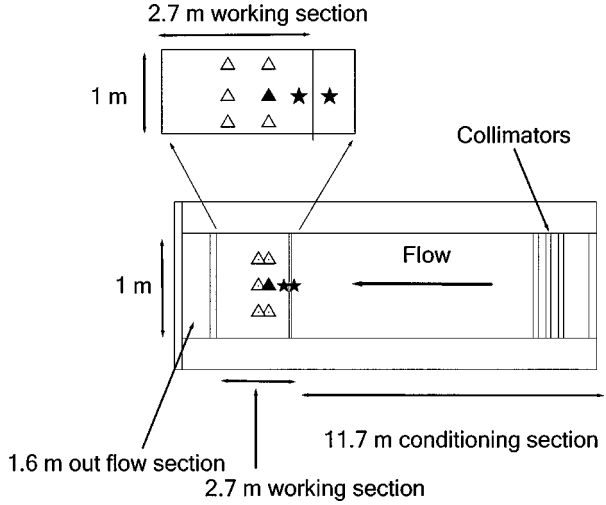

FIG. 1. Artificial stream setup at the UMBS Stream Research facility located in Pellston, MI. Water was pumped in continuously from the nearby Maple River. △—ADV measurement sites, ▲—ADV and IVEC-10 measurement site, ★—Odor source placement. Width of the stream is projected four times its real width relative to the length. The inset shows the working section. Odor sources are 30 cm apart with the most upstream source behind the working section grating. The ADV recording sites were 1 and 1.3 m from the top of the working section and the IVEC sampling site is 40 cm from the odor source.

METHODS AND MATERIALS

Animals. Male and female *Orconectes virilis* were collected from Maple Bay in Burt Lake during the summer of 2001. Crayfish were housed in flow-through outdoor metal troughs located at the University of Michigan Biological Station Stream Research Facility. They were allowed to acclimate to their new surroundings for at least 24 hr and fed on detrital material that accumulated in the holding tanks. All crayfish were released after testing into Burt Lake, downstream of the capture site, to prevent recapture on subsequent sampling trips.

Artificial Stream Setup. An artificial stream ($16 \times 1 \times 0.2$ m) was constructed of concrete cinder blocks and 4-mm plastic sheeting (Figure 1). The plume was built to recognized standards in order to form an equilibrium benthic boundary layer (Nowell and Jumars, 1987). Stream water was used directly from the Maple River, resulting in a background concentration of natural odors that was consistent throughout all experimental trials. The stream contained an 11.7-m flow conditioning section in front of the 2.7-m working section and 1.6-m outflow section. The water exited the artificial stream and re-entered the Maple River approximately

200-m downstream of the intake for the stream lab. The end of the stream was covered with a 1.5 × 0.35-m board that contained 2.54-cm diam holes evenly spaced throughout to maintain a constant depth and flow rate. Collimators consisting of three sheets of plastic egg crating (1.7-cm^2 holes) covered with fiberglass sheeting (1-mm^2 holes) were placed in the upper part of the stream to laminarize the incoming flow. The working section was partitioned off from the rest of the stream with two sheets of plastic egg crating spaced 2.7 m apart.

Water was pumped from the Maple River into a 1-m mixing area ahead of the 11.7-m stream section. In order to avoid excessive particulate matter buildup, the flow through pipes was equipped with nylon stockings secured to the ends of the flow pipe to filter out the fine organic matter and macroinvertebrate fauna.

Free stream velocity (5.0 ± 0.3 cm/sec) was measured at the beginning of each day by a Marsh–McBirney flow meter. Depth measurements ensured a uniform water depth of 20 ± 0.3 cm. The bottom of the stream was lined with cobble stones (4.5 ± 0.2 cm, $N = 24$) collected from the Maple River. To facilitate orientation responses of animals, the entire working section of the artificial stream was covered with tarps to reduce ambient light conditions.

Hydrodynamic Characteristics. The hydrodynamic characteristics of the artificial stream were determined by measuring the instream flow with an acoustic doppler velocimeter (ADV, Nortek USA). Measurements were taken at two downstream positions (1.1 and 1.3 m) within the working section of the stream. At each downstream position, three cross-stream sites and 2 depths were measured. The cross-stream sites were 51 cm from the right wall (midpoint of the stream) and 25 cm to the right and left of center of the stream (Figure 1).

For each of the vertical measurements, the probe tip was suspended at 5.9 cm and 6.5 cm above the substrate. The probe of the ADV was activated to determine the exact height above the substrate and then adjusted to the desired height. At each of the six sites (2 downstream × 3 cross-stream each at 2 heights), three-dimensional flow velocities were taken for 180 sec at a sampling rate of 25 Hz. Velocity profiles and calculation of hydrodynamic variables were performed offline (Explore package, Nortek).

Stimulus Preparation. Fish gelatin blocks were used to simulate the effect of slowly diffusing carrion odors, which crayfish feed on in natural systems. Fish gelatin was prepared by mixing 45 g of homogenized frozen ocean perch (*Perca sp.*) fillets with 28 g of Knox unflavored gelatin and 0.71 l of boiling water. After mixing, the hot gelatin was placed in a baking pan lined with plastic wrap and refrigerated overnight until solidified. Solidified gelatin was cut into $3 \times 3 \times 1.5$-cm cubes. Gelatin blocks were quartered before each trial to allow for a uniform surface area, and placed in mesh bags (1-mm^2 holes) with weights attached to the bottom to keep them in place during trials. As controls, empty mesh bags were placed in the same configuration as the bags containing the gelatin blocks.

Orientation Trials. Four male and 36 female *O. virilis* were used only once in orientation trials. Previous studies have shown that there are no differences in orientation strategies between male and female crayfish (Moore and Grills, 1999; Keller et al., 2001; Tomba et al., 2001). Crayfish were marked with reflective tape on the back of the carapace and placed inside the stream for a 20 min acclimation period. Reflective tape facilitated observations of animals in subsequent motion analysis. Following a 20-min acclimation period, the mesh bags containing the fish gelatin blocks were placed in an upstream position, 15 cm from the top grating. Bags were also marked with reflective tape to allow for visual confirmation of the crayfish finding the source. Trials were defined as successful when the crayfish came within 10 cm of the source or touched the bag itself. Trials in which the crayfish did not move, walked along the walls, or did not locate the source were discarded. Sixty crayfish were used in the experimental trials where 4 in the single 1X, 15 in the single 2X, and 11 in the dual presentations were discarded. Trials were run until the animal located the odor source for a maximum of 15 min. Trials were conducted during the months of June and July 2001 between 0800 and 1700 hours at the University of Michigan Biological Station Stream Research Facility in Pellston, Michigan. All trials were videotaped from above using a Cannon XL1 3 CCD Digital Video Camcorder. Orientation trials consisted of three experimental treatments and one control treatment. 1) Single concentration; single spatial source: One piece of fish gelatin was placed in a mesh bag at a single location within the stream ($N = 10$). Hence referred to as single 1X. 2) Double concentration; single spatial source: Two pieces of fish gelatin were placed in a single mesh bag at a single location within the stream ($N = 10$). Hence referred to as single 2X. 3) Double concentration; dual spatial source: Two pieces of fish gelatin were placed in two bags (one in each bag), one 30 cm behind the other in an upstream position. One bag was placed 15-cm downstream of the working section grating, the other 15-cm upstream behind the grating ($N = 10$). Hence referred to as a dual source. 4) Control treatments consisted of an empty mesh bag ($N = 10$). Crayfish were able to reach the more downstream source only, and were used only once in the behavioral experiments to avoid the influence of experience on orientation behavior.

Odor Characterization. To characterize the odor plume structure, gelatin was laced with a chemical tracer (0.1-M dopamine). Dopamine (3.4 g) was mixed with 7 g of Knox gelatin in 0.178 l of boiling water. Dopamine gelatin was placed in a baking dish and refrigerated overnight to solidify. The solidified cubes ($3 \times 3 \times$ 1.5-cm) were quartered, placed in mesh bags, and placed into the stream in the same positions as the fish gelatin.

An electrochemical detection technique was used to characterize the effects of different spatial arrangements of the odor source. The temporal distribution of dopamine was sampled at a rate of 10 Hz using the In Vivo Electrochemistry Computer System (IVEC-10). Electrochemical measurements were made with a

30-μm diameter carbon fiber electrode calibrated by using four concentrations of dopamine ranging from 0 to 30 μM. Electrodes were calibrated with the above concentration range in a 50-ml beaker. These concentrations were used only for calibration of the electrode. Electrode readings showed linearity over this range (coefficient of determination: $r^2 > 0.97$). Five-min electrochemical recordings that used 1-M dopamine gelatin were made 40 cm from the odor source with each source arrangement to measure the temporal and spatial distribution of the chemical double, hence food odor distribution (Figure 1).

Data Analysis. Flow data obtained through measurements by using the ADV system were analyzed by the commercial software program provided with the ADV system (Explore). Hydrodynamic characteristics were estimated from the data following standard practices (Sanford,1997). The turbulent energy dissipation rate was calculated from Eq. 1 below (Sanford, 1997):

$$\varepsilon = (U^3)/\kappa z \tag{1}$$

where U is the free stream velocity, κ is von Karman's constant (0.41), and z is the height above the stream bed.

From the values obtained, we were able to calculate the Kolmogorov microscale (η, Eq. 2) and the Batchelor microscale (η_s, Eq. 3; Sanford, 1997). These microscales are the measurements of the smallest eddy sizes, Kolmogorov, and the smallest distances for differences in chemical concentrations, Batchelor.

$$\eta = 2\pi(v^3/\varepsilon)^{1/4} \tag{2}$$

$$\eta_s = 2\pi\left(v D_s^2/\varepsilon\right)^{1/4} \tag{3}$$

The coefficient D_s is the molecular diffusion coefficient of dopamine (2×10^{-5} cm^2/sec). A spectral analysis of the velocity values was performed using the ADV Explore program at a 95% confidence level using Tukey HSD comparison test.

IVEC-10 data were analyzed by using an in-house basic program. Spatial and temporal components of the odor plume such as, maximum height of the odor pulse (highest concentration of chemical within the odor pulse), absolute slope (from threshold to highest concentration within an odor pulse/rise time), maximum slope (highest increase of concentration between two measurements), and rise time (from threshold to the highest value within an odor pulse), number of peaks (number of odor pulses in the entire trial), intermittency (time between peaks of two odor pulses), and spectral density (energy of the signal as a function of frequency) were measured for each arrangement of the odor sources. The beginning of odor pulses was determined as the concentration rose above a threshold, and the end was determined when the concentration dropped below 30% of the previous peak height (Moore and Atema, 1991). Values obtained were averaged for each spatial position and analyzed using a Kruskal–Wallis ANOVA by ranks test. Tests for normality of

the data showed that they were not normally distributed. Spectral analysis of odor signals (FFT and spectral densities) was performed with a commercial statistical package (Statistica by Statsoft). For each odor source measurement, there were 3432 data points. These were subsequently divided into five subsamples for each of the odor arrangements. The subsequent subsamples were used in the spectral analysis for each odor placement (Moore et al., 2000). For statistical analysis only, the energy in these odor spectra were binned in 0.5-Hz bins. A 2-way factorial ANOVA, with odor arrangement and frequency bin as the factors, was performed with the five-subsample (686 data points each) spectra from each odor arrangement. A Tukey HSD *post hoc* test was used to determine individual differences. The resulting spectra from the five subsamples of each odor placement were averaged and plotted against frequency.

Videotaped trials were digitized 1 frame/sec by using the Peak Motus System to obtain X, Y spatial coordinates of crayfish movements throughout each trial. An array of behavioral parameters were analyzed including success rate, time to locate odor source, linearity of path, walking speed, walking speed toward source, heading angle, heading angle relative to source and relative to upstream, and turning angle. These are explained and defined in a previous publication (Moore and Grills, 1999). A detailed temporal analysis was performed on walking speeds, turning angles, and heading angles. These were analyzed statistically using a MANOVA and Fisher LSD *post hoc* test. The behavioral parameter of question (i.e., walking speed, turning angle, and heading angle) was averaged over a 10-cm bin at different distances from the odor source for each individual animal. These individual binned averages were then used in either a 2nd (walking speed) or 3rd (turning angle) order polynomial regression analysis (Moore et al., 1991). Each of these regressions was statistically significant ($P < 0.05$). The coefficients for each of the individual regressions were averaged for the entire population and used to generate the population level regressions displayed on the figures in this manuscript. Only those animals that did not contact the side walls during any part of the orientation trial were included in the regression analysis. Following regression analysis, walking speeds were analyzed in blocks of distance from the odor source; 100–150 cm, 50–100 cm, and 0–50 cm, by using a 2-way ANOVA and unequal *N* HSD post hoc test.

RESULTS

Hydrodynamic Characterization. Measurement of the hydrodynamic structure of the artificial stream showed that the boundary layer was in equilibrium throughout the working section of the stream (Table 1). Spectral analysis of both the 1.1-m and 1.3-m sites at each vertical point indicated a uniform consistency of flow speed (Figure 2), i.e., the flow speed did not change significantly over the course of the working section.

TABLE 1. HYDRODYNAMIC CHARACTERISTICS OF AN ARTIFICIAL STREAM[a]

Distance from top of working section	U_∞ (cm/s)	ε (cm^2/s^2)	η	η_s
1.1 m	6	0.09	1.76E-05	7.04E-11
1.3 m	6	0.09	1.79E-05	7.17E-11

[a] Abbreviations: U_∞ = free stream velocity, ε = turbulent energy dissipation rate, η = Kolmogorov scale, and η_s = Batchelor scale.

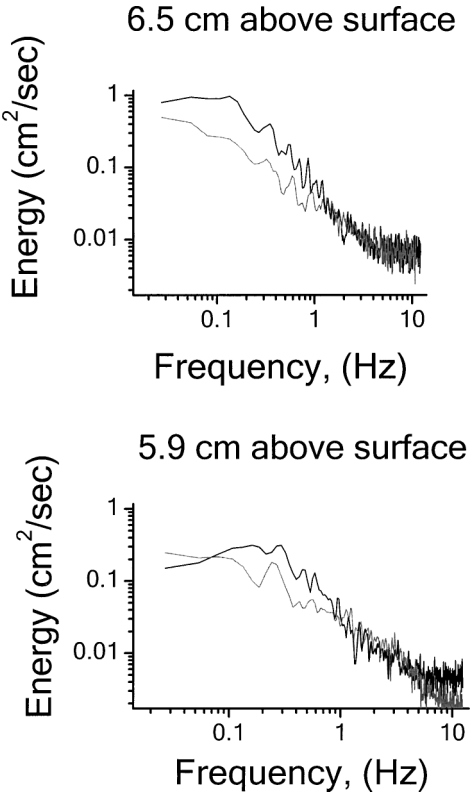

FIG. 2. Spectral analysis of water velocity in the artificial stream at two points within the working section of the stream. The solid black line represents measurements taken 1.1 m from the top of the working section and the light solid line represents measurements taken 1.3 m from the top of the working section. The vertical profile of the stream velocity at both distances shows the water velocity has reached equilibrium and that the boundary layer is 5.9 cm above the substrate.

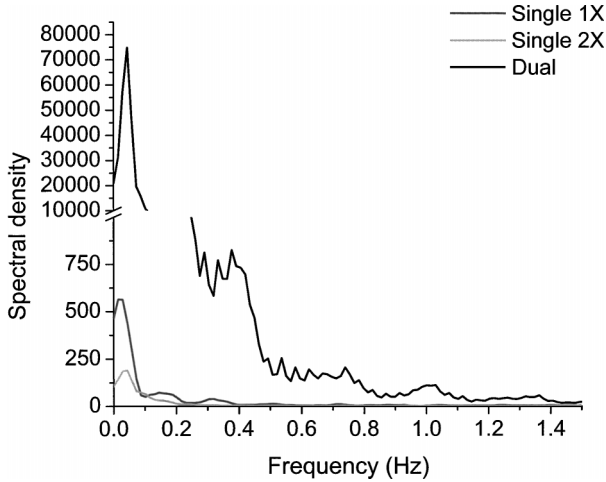

FIG. 3. Spectral analysis of the odor plume structure of the single 1X ($N = 1716$), single 2X ($N = 1716$), and dual ($N = 1716$) odor sources using 0.1 M dopamine gelatin as an odor source. The light solid lines represent the single source arrangements and the black solid line represents the dual source arrangement.

Odor Characterization. Spectral analysis of the dual source arrangement with 1-M dopamine gelatin showed changes in the temporal patterns of the odor plume. The dual source arrangement exhibited a wide range of signal fluctuations across a wider range of frequencies (Figure 3). Analysis of the spectra of the different odor sources showed that there was an interaction between the placement of the odor sources and the frequency of the peaks (2-way ANOVA: $F_{18} = 14.9$, $P < 0.001$). *Post hoc* analysis showed that the dual source had more energy at the lower frequency peaks compared to the single 1X and single 2X sources (Tukey HSD: $P < 0.001$). There were no differences between the single 1X and single 2X sources ($P = .99$). Spectral analysis of both single source arrangements showed lower frequency signals (<0.15 Hz) with a steady decrease in signal energy as frequency increased. There was an overall difference in the intermittency of peaks within the three odor arrangements (Kruskal–Wallis: $H_{2,1004} = 90.91$, $P < 0.05$). Figure 4 shows that the intermittency between pulses was smaller for the dual source compared to the single 2X arrangement, but the single 1X arrangement had shorter intermittency than the other spatial arrays.

Table 2 shows additional changes in the odor plume structure as a result of altered spatial arrangement. The concentration or maximum height of the chemical tracer within each odor patch increased with the dual source arrangement (Kruskal–Wallis: $H_{2,1004} = 55.72$, $P < 0.05$). The rise time decreased with the dual source arrangement (Kruskal–Wallis: $H_{2,1004} = 19.1$, $P < 0.05$). Additionally,

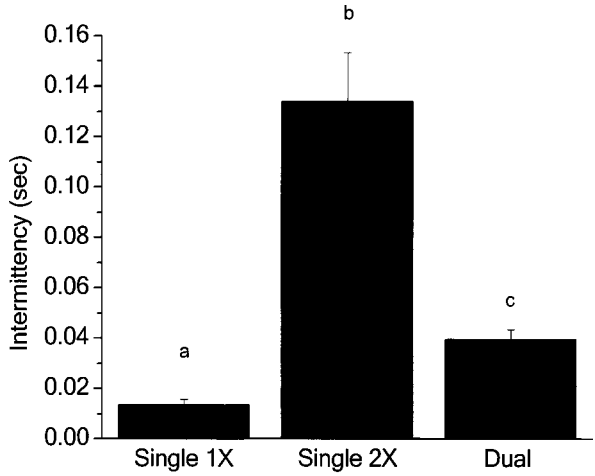

FIG. 4. Average time (± SEM) between signal peaks (intermittency) of 0.1 M dopamine gelatin. The dual odor source arrangement had less time between odor pulses than the single 2X source. Columns with the same letter are not significantly different from each other. Significant differences were determined by a Kruskal–Wallis ANOVA by ranks and multiple comparisons: single 1X ($N = 453$) versus dual ($N = 350$) ($Z = 4.32$, $P < 0.001$), single 2X ($N = 200$) versus dual ($Z = 2.96$, $P < 0.009$).

the absolute slope (Kruskal–Wallis: $H_{2,1004} = 149.3$, $P < 0.05$) and maximum slope (Kruskal–Wallis: $H_{2,1004} = 108.13$, $P < 0.05$) of the odor patches increased as a result of the dual source.

Quantitative Analysis of the Temporal Aspects of Orientation Behavior. The temporal and spatial aspects of odor tracking behavior revealed differences in the strategies used to orient upstream. As crayfish moved upstream there was an overall effect of odor placement on spatial orientation (MANOVA: *Rao's* $R_{12,87} =$

TABLE 2. CHARACTERIZATION ODOR PLUME STRUCTURE OF SINGLE 1X AND SINGLE 2X AND DUAL ODOR SOURCE ARRANGEMENTS

	Single 1X	Single 2X	Dual
Maximum height	1.87	1.91	3.05*
Absolute slope	5.27	4.33	8.28*
Maximum slope	10.08	9.28	13.71*
Rise time	0.45	0.63	0.38*

*Indicates significant differences (Kruskal–Wallis, $P < 0.001$). Valid cases varied from 452 for single 1X, 200 for single 2X, and 350 for dual.

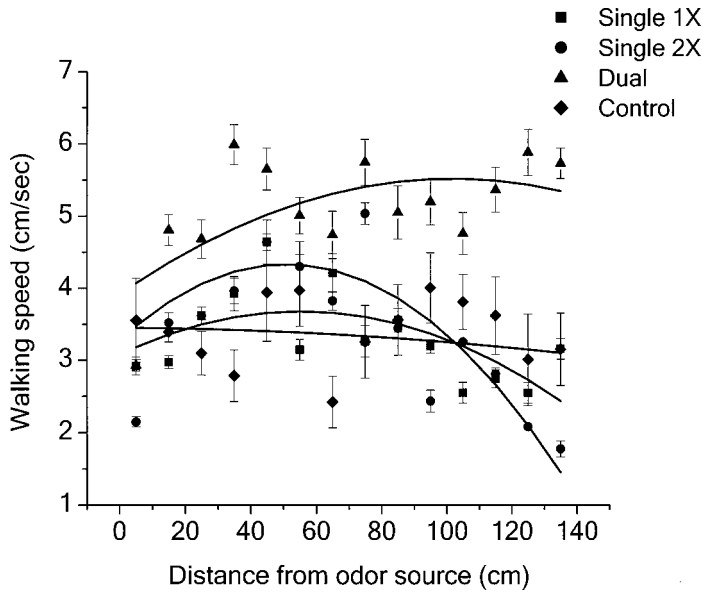

FIG. 5. Polynomial regression of walking speeds of crayfish as a function of distance to the odor source. ■—single 1X ($N = 7$); ●—single 2X ($N = 5$); ▲—dual ($N = 7$); ♦—control ($N = 10$).

2.83, $P < 0.05$). Turning angles of crayfish as they moved toward the source were smaller when presented with the dual source (46.76 ± 6.20 degrees) than control crayfish (67.01 ± 6.71 degrees) (LSD: $P < 0.05$). Compared to the single 1X and single 2X source arrangements, there were no significant differences when crayfish were presented with the dual source (LSD: $P = 0.16$). There were no differences between control crayfish and either single source arrangement (LSD: $P = 0.65$). Crayfish presented with the empty bag controls did not locate the empty bags in the 15-min time frame allowed. As for the heading angle and the net-to-gross measurements, there were no differences between the odor placements.

Analyzing walking speeds and turning angles as a function of distance to the source revealed significant ($P < 0.05$) and different regressions for the different odor treatments. Walking speeds as a function of the distance to the source showed a three phase pattern. This general pattern was identical between the two single location odor sources (single 1X and single 2X source treatments). Under the dual odor treatment, the temporal aspects of orientation behavior were altered (Figure 5.). Crayfish presented with the dual source walked faster when they were 100–50 cm away compared to the single 1X, single 2X, or control presentation (HSD: $P < 0.05$). As they approached the odor source, between 0–45 cm, the differences in walking speeds under the three treatments were not maintained

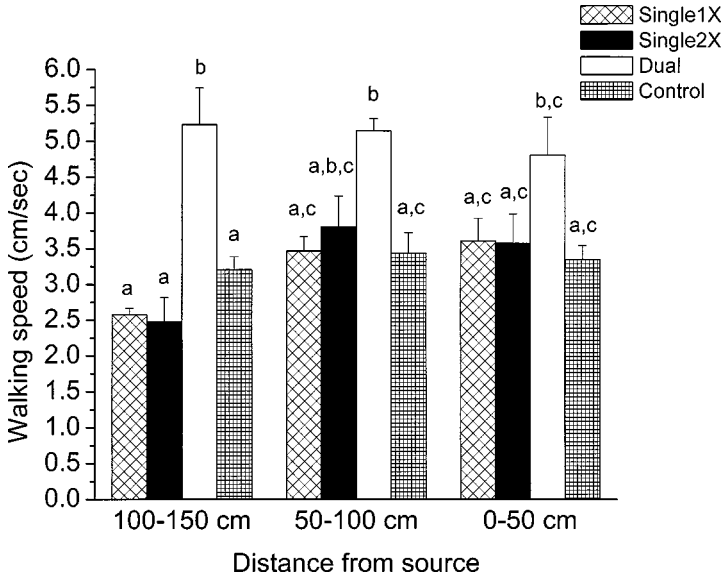

FIG. 6. Comparison of walking speeds of crayfish (± SEM) when presented with the different odor placements in blocks of 50-cm distances from the odor source. $N = 10$ for each treatment. Columns with the same letters are not significantly different from each other. A 2-way ANOVA for distance to the odor source and odor placement was performed ($F_{2,2} = 31.5, 4.0$). Unequal N HSD: $P < 0.05$.

(Figure 6). This suggests that the placement, not the concentration, of the odor sources had an affect on temporal aspects of orientation behavior.

Turning angles of animals as a function of the distance to the source showed a similar result, in that animals started searching using identical turn angles far away from the source and then increased their turning angles sharply as they approached the source (Figure 7). Both single source arrangements showed a similar pattern in turn angles with relation to distance from the source, whereas the dual source arrangement had a much different pattern, particularly at a greater distance from the odor source (Figure 7). Turn angles of crayfish presented with the dual source were more constant as they moved upcurrent as opposed to the single source arrangements.

DISCUSSION

In order to understand how crayfish respond to complex signals we must know how those signals are distributed in a flowing environment. In the present study, IVEC measurements showed that spatially separated odor sources caused

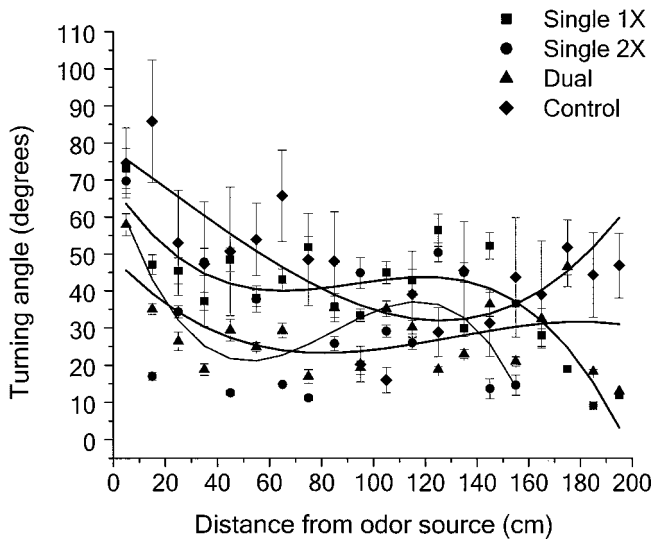

FIG. 7. Polynomial regression of turning angles of crayfish as a function of distance from the odor source. ■—single 1X ($N = 7$); ●—single 2X ($N = 5$); ▲—dual ($N = 7$); ♦—control ($N = 10$).

the odor plume to have higher frequency components and more energy in the lower frequency components (Figure 3). We have defined the higher frequency components and increased energy as an increase in the "complexity" of the odor signals perceived by the crayfish during orientation. The spatial and temporal structure of the plume was more "complex" in the dual source arrangement compared to both single source arrangements. This "complexity" was further reflected in other temporal aspects of the odor signal such as mean peak height and intermittency (Table 2, Figure 8). The dual source arrangement presented crayfish with larger concentration fluctuations in shorter pulses than the single 2X source even though they were presented with similar surface area and concentration. The pattern in intermittency with the different spatial arrays showed that the single 1X source had more peaks and smaller intermittency between these peaks than either of the double sources.

Receptor cells of lobsters have been shown to act as temporal filters to extract spatial information from fluctuations in odor plumes (Gomez and Atema, 1999). These receptor cells vary in their temporal filtering capabilities, leading to differences in response to differing temporal fluctuations in odor plumes (Gomez et al., 1994; Gomez and Atema, 1996, 1999).

The behavioral data in the present study indicate the differences in the temporal complexity of the odor signal that resulted in more differences in the temporal

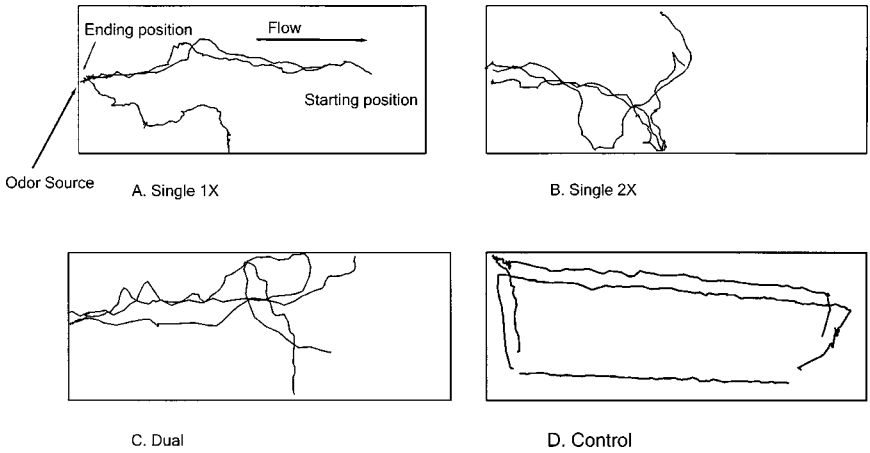

FIG. 8. Representative orientation paths of three crayfish when presented with, A—single 1X, B—single 2X, C—dual, and D—control. Direction of flow, the relative position of the odor sources, and crayfish starting and ending positions are shown in A. Tracks were recorded 1/sec.

aspects of orientation behavior (walking speed, and speed toward source) than the spatial aspects of orientation behavior (turning and heading angles). In general, crayfish located the odor source in all treatments. The paths of crayfish were similar to previously published studies and lacked any zig-zag patterns found in male moth orientation studies (Mafra-Neto and Cardé, 1996). In addition, orientation paths looked similar to those published for lobsters (McLeese, 1973; Moore et al., 1991). There were periods in the paths where animals stopped, moved sideways and backwards, as well as toward the odor source (Figure 5). Qualitatively, the orientation paths looked similar among the treatment groups. The orientation paths showed that animals exhibited a fairly straight course toward the source with a few corrections as they moved upcurrent.

By linking the IVEC results with the behavioral results, we show that the temporal dynamics of odor fluctuations were guiding upcurrent movement and turning behavior of crayfish. By altering the spatial arrangement of the odor sources we were able to alter temporal characteristics of the signal structure without altering the temporal dynamics of hydromechanical signals. Thus, changes seen in orientation strategies were a result of changes in the spatial arrangement of a food source as opposed to an increase in concentration of the individual food sources, or any alteration in mechanical information. None of the behavioral measurements quantified in this system were altered by changes in the concentration of the odor (single 2X vs. single 1X). When one source was split and separated in space, crayfish maintained a faster walking speed and exhibited more consistent turning

behavior as they moved upcurrent (Figures 5–7). In the current design, as crayfish reached approximately 50 cm from the odor source, turning angles increased sharply and walking speeds remained constant regardless of source placement. This change in behavior may indicate the point where animals switch to a local search strategy, possibly using more proximate cues, visual or tactile, to locate a close source. This general theme has been demonstrated in other studies with lobsters (Moore et al., 1991). As they approach a certain distance to the source a local search strategy takes over. The exact distance at which the local search occurs will change with changes in design paradigms, such as flow speed, turbulence, or concentration of the source.

Ecologically, aquatic organisms are faced with several problems that they must solve in order to perform life sustaining behaviors that are triggered by chemical information. In typical habitats, these animals experience multiple odor plumes that are mixing over a multitude of physical habitats (Finelli et al., 2000; Moore et al., 2000). Faced with this complexity, animals must not only recognize and distinguish predator from food, mate from food, or food from other odor signals, but also must be able to have some special awareness of these multiple odor sources, i.e., the ability to discriminate where these sources lie in relation to each other. With even a primitive ability to distinguish locations of odor sources relative to each other, animals will be able to make choices regarding foraging, avoiding predators, or mating. Studies with crayfish showed that they can effectively locate the spatial relationship of conflicting odor cues (Tomba et al., 2001). The results of the present study suggest that crayfish alter their behavioral output in response to increased temporal stimulation, which in turn, is a result of the changed spatial arrangement of food sources.

The way Crayfish orient toward an odor source is affected by changes in intermittency and intensity of the signal within each odor patch arriving at the antennules (Kozlowski et al., 2003). In studies with moths, the fine-scale structure, the flux of the stimulus, and the wind direction are all important in determining the response of males to female pheromones (Vickers and Baker, 1992, 1994; Mafra-Neto and Cardé, 1996). Changes in the rate of release and the fine-scale structure of the plume alter male flight characteristics in response to these changes (Murlis and Jones, 1981; Vickers and Baker, 1992) For blue crabs, the structure of the plume, as well as the direction of water flow, is important in orientation to odor sources (Zimmer-Faust et al., 1995; Finelli et al., 2000). This suggests that blue crabs orient by utilizing both the spatial and temporal aspects of an odor plume. Although it has been claimed that these animals are using an odor-gated rheotaxis, a recent model results make it hard to pinpoint an exact orientation mechanism for blue crabs (Weissburg and Dusenbery, 2002). Our findings lend support to the idea that temporal stimulation of the animal may play an important role in orientation behavior. We hypothesize that the presence or absence of the odor signal mediates movement toward the odor source. By altering the temporal and spatial dynamics

of odor plumes we see changes in the temporal and spatial behavioral patterns of crayfish. Thus, we suggest that some spatial aspects of the odor plume are critical in changing the turning angles exhibited by crayfish during orientation behavior.

In summary, both the spatial and temporal aspects of orientation behavior in crayfish were altered by changes in odor plume dynamics. With an increase in the temporal complexity of odor signals, crayfish responded with higher walking speeds and smaller turning angles. By combining the chemical characteristics of the odor plume with hydrodynamic and behavioral measurements, we hypothesize that these crayfish were using a combination of the classically defined kinesis and taxis. Some aspects of crayfish orientation behavior can be termed a kinesis in that walking speeds were controlled by the stimulus distribution, while other aspects were similar to a taxis in that turning angles were decreased in the presence of increased temporal stimulation. It appears as if the crayfish in this study were not performing an odor-gated rheotaxis that has been reported for other benthic crustaceans (blue crabs, Weissburg and Zimmer-Faust, 1994).

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CHEMO-ORIENTATION USING CONSPECIFIC CHEMICAL CUES IN THE STRIPE-NECKED TERRAPIN (*Mauremys leprosa*)

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Abstract—Although chemical communication has been studied intensively in many reptilian species, little attention has been paid to the role that chemical signals play in aquatic reptiles, such as freshwater turtles. Here, I tested the hypothesis that the stripe-necked terrapin (*Mauremys leprosa*), an abundant freshwater turtle that inhabits the Iberian peninsula, is able to recognize chemical cues from conspecifics in the water and to modify its behavior in response to such cues. I compared the time spent by adult males and adult females in clean water to the time spent in water that presumably contained their own odor, odor from other males, and odor from other females, both during and outside the mating season. Results show that outside the mating season, both males and females avoid water that contains chemical cues from conspecifics of the opposite sex. During the mating season, male turtles clearly select water with chemical cues from females. Moreover, males prefer to occupy water from their home containers over clean water, and avoid water with chemical cues from other conspecific males. Conversely, during the mating season, females prefer to occupy water with chemical cues from other females, but do not select water from their home containers or water from males. The evolution of chemical communication in turtles, its relation to sexual selection processes, and the implications for turtle behavior are discussed.

Key Words—Chemical communication, chemo-orientation, *Mauremys leprosa*, pheromones, sexual selection, turtles.

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INTRODUCTION

The study of chemical communication is recognized as an integral part of research on reptilian social behavior (Mason, 1992). Although communication by chemical cues has been known in Chelonian species for a long time (Neill, 1948; Legler, 1960; Rose, 1970), little attention has been paid to the role that chemical signals play in turtle behavior in comparison to other groups of reptiles such as lizards (e.g., Alberts and Werner, 1993; Cooper, 1994; Aragón et al., 2001; López and Martín, 2002; López et al., 2002) or snakes (e.g., Begun et al., 1988; Chiszar et al., 1990; LeMaster et al., 2001).

Chelonians have well-developed nasal olfactory and vomeronasal systems (Halpern, 1992; Hatanaka and Matsuzaki, 1993), and these functional chemosensory systems may be used for mediation of turtle behavior (Mason, 1992; Graham et al., 1996; Quinn and Graves, 1998). In a variety of chelonians, behavioral responses to chemical cues occur in diverse social contexts, including aggregation, aggression, and sexual behavior (reviewed in Mason, 1992; Alberts et al., 1994; Graham et al., 1996; Quinn and Graves, 1998). For example, Rose (1970) suggested that chemicals play a role in aggressive encounters between male tortoises and may be important in mediating courtship behavior. Eisner et al. (1977) showed that secretions from the axillary glands of *Sternotherus* may serve a dual function. These secretions are involved in antipredator mechanisms, whereas during the mating season, the glandular contents serve as sex-recognition cues by which courting males discriminate females. Chemicals may also play an important role in turtle homing behavior, home area recognition, and orientation (Grassman, 1993; Graham et al., 1996). Homing by using chemical cues from conspecifics was demonstrated by Quinn and Graves (1998) in the painted turtle (*Chrysemys picta*). The results indicate that *C. picta* discriminate between chemical cues from home ponds and other ponds. Moreover, the response of males and females to the factors mediating home pond recognition is sexually dimorphic. Female *C. picta*, but not males preferred to occupy water from home ponds over water from other ponds that contained conspecifics. In *Testudo hermanni*, experimental elimination of olfactory function resulted in impaired homing ability and a 60–70% reduction in reproductive behavior (Chelazzi and Delfino, 1986).

Behavioral studies on chemical communication in freshwater turtles are particularly scarce (see Mason, 1992), although chemical communication has been shown to be widespread among aquatic organisms (Liley, 1982; Dodson et al., 1994; Aragón et al., 2000). Underwater, olfactory detection could be especially important when visual cues are limited. Most freshwater turtles have several specialized glands in the skin, called Rathke's glands, which secrete chemical components into the environment (Ehrenfeld and Ehrenfeld, 1973). Although the function of these gland secretions remains unknown, and the substances are not well

characterized, they may serve as signals in social interactions, like chin gland secretions of some terrestrial tortoises (Alberts et al., 1994). Moreover, in several species of freshwater turtles, feces may serve, alone or in combination with glandular secretions, as a source of chemical components (Harless, 1979; Mason, 1992).

The stripe-necked terrapin, *Mauremys leprosa* (Schweiger, 1812) is a riverine emydid turtle that inhabits the center and south of the Iberian Peninsula (Andreu and López-Jurado, 1998). It seems that olfactory communication could be particularly important in this species for many reasons: (1) It often inhabits quiet and troubled waters (unpublished data), as *M. caspica rivulata* does (see Gasith and Sidis, 1984), where dense vegetation and turbidity can make visual cues unreliable, thus favoring chemical detection of conspecifics. (2) It possesses a pair of inguinal Rathke's glands (Loveridge and Williams, 1957; Ehrenfeld and Ehrenfeld, 1973), whose secretions have an intense odor. (3) Sniff behavior is a frequent and conspicuous habit in *M. leprosa*, as in other turtle species (Ernst, 1971; Seigel, 1980; Kramer and Fritz, 1989; Kaufmann, 1992). The mating system of the stripe-necked terrapin is not well characterized, but the mating season occurs in spring (unpublished data), as in most temperate-zone turtles (Moll, 1979). During summer (July–August), activity of *M. leprosa* is particularly low because many creeks in central Spain dry up partially or completely.

Here, I report on a 2-year laboratory experiment that tested the hypothesis that *M. leprosa* is able to recognize chemical cues from conspecifics in water. I compared time spent by male and female turtles in clean water vs. time spent in water presumably containing their own odor, odor from other males, and odor from other females, both during and outside the mating season. Results showed that preferences for different types of water vary between sexes and between seasons.

METHODS AND MATERIALS

Study Animals. I captured a total of 32 individuals of *Mauremys leprosa* during two consecutive years of experimental study. In the first year (2001), experiments were carried out outside the mating season of the species. In July, once the mating season had ended, I captured 16 turtles, eight adult males and eight adult females of similar size (Males: carapace length $\bar{X} \pm \text{SE} = 150.4 \pm 3.1$ mm, range: 132–160 mm; weight $\bar{X} \pm \text{SE} = 386.2 \pm 26.6$ g, range: 240–460 g. Females: carapace length $\bar{X} \pm \text{SE} = 180.9 \pm 3.9$ mm, range: 169–205 mm; weight $\bar{X} \pm \text{SE} = 762.5 \pm 52.2$ g, range: 610–1070 g). In the second year (2002), I carried out the same experiments as in 2001, but in this case during the mating season of the species. Another 16 turtles, eight adult males and eight adult females of similar size, were captured in May, which coincided with the turtle mating

season in their original natural populations (Males: carapace length $\bar{X} \pm \text{SE} = 156.5 \pm 2.5$ mm, range: 145–166 mm; weight $\bar{X} \pm \text{SE} = 390.3 \pm 27.2$g, range: 245–498 g. Females: carapace length $\bar{X} \pm \text{SE} = 179.6 \pm 5.2$mm, range: 153–200 mm; weight $\bar{X} \pm \text{SE} = 756.7 \pm 50.3$g, range: 608–1030 g).

To ensure that turtles had not been in previous contact, which might have affected the outcome of the experiments, in both years I captured them in several creeks over the same large area (more than 1000 km^2) in the Toledo province (central Spain). Turtles were hand-captured or creel-captured using sardines as bait, under licence from the Consejería de Medio Ambiente de Toledo. They were individually housed in containers ($60 \times 40 \times 20$ cm) filled to a depth of 15 cm with water, and located in outdoor conditions. They were fed twice per week with dead fish and chicken liver, and were held in their home-containers for at least 10 d so that they would become familiarized with their new environment prior to testing. At the end of experiments, turtles had been in captivity for 30 d. All animals were healthy during the trials and were released at the capture sites at the end of the experiments.

Experimental Design. I conducted the experiments under outdoor conditions using experimental aquaria consisting of two plastic containers ($73 \times 57 \times 17$ cm) joined by two ramps, which made it possible for a turtle to cross easily from one container to the other. Each turtle was given the choice between two containers that had been previously filled with water from different origins. I performed three types of trials for each turtle; it was allowed to stay in (1) clean water vs. water from its own home-container, (2) clean water vs. water from one home-container of an individual of its own sex, and (3) clean water vs. water from one home-container of an individual of the opposite sex. Water with chemical scents of individual turtles used in each test was taken from the home-containers in which each turtle had been maintained for 3 d. I filled home containers of the "odor donor turtles" with 20 l of clean water, and during these 3 d, other unoccupied containers were filled with 20 l of clean water. Trials started at 1030 GMT when water (clean and stimulus) was poured into the experimental aquaria, and the experimental turtles were located between the two ramps in a neutral position not biased towards any container. Turtles were manipulated by using fresh gloves to avoid odor contamination. Aquaria were covered with pieces of opaque fabric, 2 m in height, in order to avoid solar or visual cues that might affect orientation during trials. I checked during each trial that the water temperature was similar in both containers, and leveled the aquaria to control possible effects of positive geotaxis (DeRosa and Taylor, 1980). I randomly chose which male or female was used in each trial, and the side in which clean water was placed was also randomized within a treatment. Each turtle participated in only 3 trials (clean vs. its own odor, clean vs. odor from another turtle of its own sex, and clean vs. odor from another turtle of the opposite sex). The three trials were spaced at least 6 d apart to avoid

turtle-stress during the experiments. At the start of each trial, turtles typically explored the two containers, frequently entering the water of one and changing to the other, then remaining in one and occasionally changing to the other. Each trial lasted 8 hr (from 1100 to 1900 GMT). I used the instantaneous scan sampling method; turtles were monitored every 20 min from a hidden point, recording their location in the experimental aquaria. Thus, the locations observed on each of the 24 scans were considered to be representative of a turtle's use of space in the aquaria. In each scan, if a turtle was situated in either of the two containers, it was designated as having chosen that container, whereas if it was located out of the water (i.e., on the ramps), it was designated as having made no choice. At least two recordings in each container within the first 2 hr were considered necessary for a trial to be valid, in order to ensure that individuals were exposed to both samples of water. After each trial, the containers were drained and thoroughly rinsed with clean water and odorless soap to avoid odor contamination. At the end of trials, I determined the choice of a given turtle based on the time spent in each container (see Aragón et al., 2000). I considered that an experimental turtle had chosen the water placed in one of the two containers if it spent more than 60% of its time (excluding time spent in the no-choice area) in that container, whereas if the percentage of time spent in any container was between 40 and 60%, I considered that the turtle had had no preference for either container. At the end of the trials, each turtle was classified as having chosen the container filled with clean water, the container filled with stimulus water, or as having shown no preference for either container. To assess the preference of a group of turtles, I calculated the number out of the total that preferred a particular stimulus (i.e., spent greater than 60% of the time) and compared it to an expected binomial distribution, assuming frequencies to be equiprobable on each side (for a similar procedure see Chivers et al., 1997; Aragón et al., 2000). If the distribution of individuals that preferred one container or the other deviated significantly from the expected binomial distribution, that group was considered to have chosen one sample of water. Previous analyses demonstrated that there are no preferences between two clean-water tests.

RESULTS

Results show that preferences for different types of water vary between sexes and between seasons. Table 1 shows the number of turtles that spent greater than 60% of their time in either container for each treatment outside the mating season, and the corresponding *P* values from two-tailed Binomial tests. Outside the mating season, both males and females significantly avoided water that contained chemical signals from individuals of the opposite sex, but they had no preferences for water

TABLE 1. TIME SPENT BY MALES AND FEMALES OF *M. Leprosa* IN CONTAINERS OUTSIDE THE MATING SEASON

	Clean water vs.		
Outside the mating season	Own water	Male water	Female water
Males[a] (clean/other)	2/6	6/2	8/0
P value	0.289	0.289	0.008
Females[a] (clean/other)	6/1	7/0	4/3
P value	0.125	0.015	0.99

[a]The number of turtles that spent >60% of their time in each container for each treatment (clean water vs.: own water/water from a male/water from a female), and the corresponding *P* values from two-tailed Binomial tests are indicated.

from their home-containers or water with chemical cues from individuals of the same sex (Table 1). Table 2 shows the number of turtles that spent greater than 60% of their time in either container for each treatment during the mating season, and the corresponding *P* values from two-tailed Binomial tests. During the mating season, males responded actively to the different water types. They clearly selected water with chemical cues from female turtles (Table 2), but they preferred water from their home-container over clean water, and avoided water with chemical cues from other males (Table 2). Conversely, females preferred water with chemical cues from other females during the mating season, but did not choose water from their home-containers or water from males (Table 2). Figures 1 and 2 show differences in responses to chemicals of both males and females during and outside the mating season, highlighting the preference of males for female odors during the mating season and the avoidance of such odors outside the mating season.

TABLE 2. TIME SPENT BY MALES AND FEMALES OF *M. Leprosa* IN CONTAINERS DURING THE MATING SEASON

	Clean water vs.		
During the mating season	Own water	Male water	Female water
Males[a] (clean/other)	0/7	7/0	0/7
P value	0.016	0.016	0.016
Females[a] (clean/other)	4/4	3/3	0/6
P value	0.99	0.99	0.031

[a]The number of turtles that spent >60% of their time in each container for each treatment (clean water vs.: own water/water from a male/water from a female), and the corresponding *P* values from two-tailed Binomial tests, are indicated.

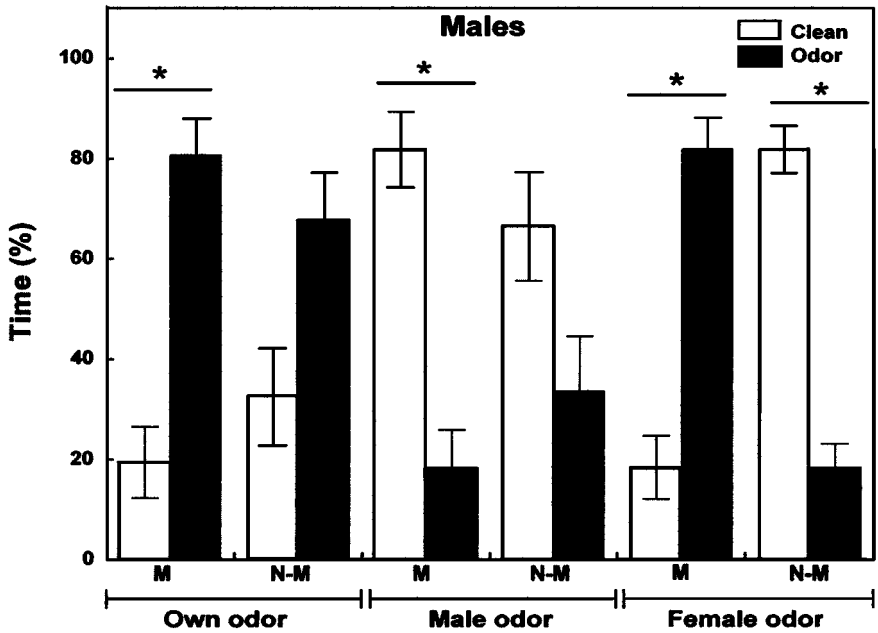

FIG. 1. Comparison of the time percentage spent by males in the containers for each treatment (□ clean water; ■ odor water) in the mating (M) and non-mating (N-M) seasons. Signed (*) treatments indicate trials in which turtles significantly chose one type of water.

DISCUSSION

Four functions of movements by turtles have been documented: feeding, mating activities, basking, and hibernation or hiding (Gibbons et al., 1990). Numerous environmental cues such as temperature, water current, visual cues, the earth's magnetic field, or positive geotaxis have been implicated in the mediation of turtle movements and turtle orientation (Casteel, 1911; Sexton, 1959; Emlen, 1969; DeRosa and Taylor, 1980; Yeomans, 1995; Lohmann et al., 1999). Chemical cues from conspecifics may also play a role in the mediation of such movements and behaviors, as indicated by this study. Outside the mating season, when mating activities of freshwater turtles do not occur, access to different resources such as food or basking sites may result in aggressive interactions between the sexes (Rovero et al., 1999). Avoidance of these kinds of interactions could be the reason that males and females tend to avoid water with chemical cues from individuals of the opposite sex outside the mating season. In other turtles, competition for food between males and females has evolved sexual differences in head proportions promoting niche divergence (Lindeman and Sharkey, 1999). In *M. leprosa*, there

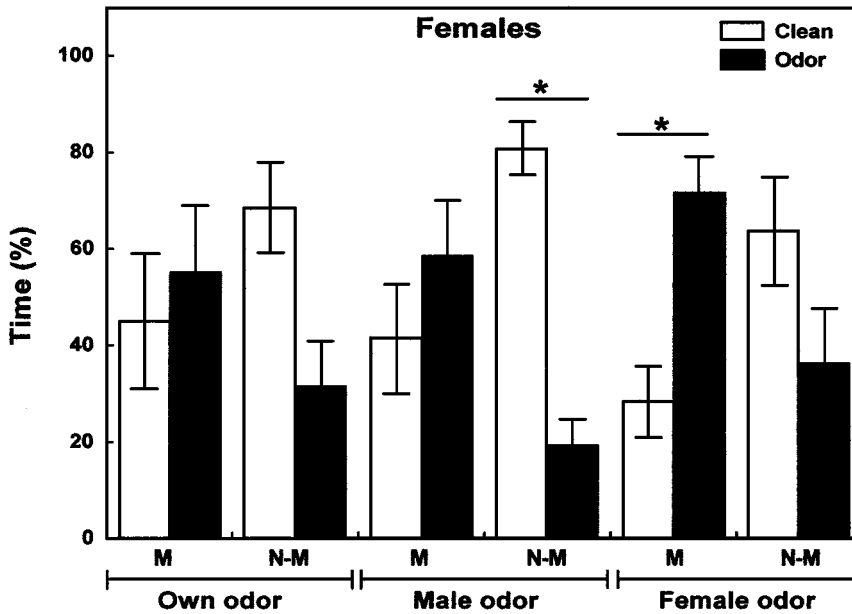

FIG. 2. Comparison of the time percentage spent by females in the containers for each treatment (□ clean water; ■ odor water) in the mating (M) and non-mating (N-M) seasons. Signed (*) treatments indicate trials in which individuals significantly chose one type of water.

is no evidence that niche divergence or variation in head proportions occurs (unpublished data). Thus, spatial segregation of the sexes could be a means whereby males and females avoid competition for resources outside the mating season.

The results suggest that males of *M. leprosa* are able to identify chemical cues from females in the mating season. During courtship and mating, extensive male sniffing and head bobbing have been reported in apparent response to female gland secretions in a number of turtles, e.g., *Gopherus berlandieri*, *Geochelone radiata*, or *Clemmys insculpta* (Rose, 1970; Auffenberg, 1978; Kaufmann, 1992). Male *M. leprosa* probably use female scents to detect and locate prospective mates by chemotaxis when vision is limited. Berry and Shine (1980) suggest that mate-searching is important for freshwater male turtles, and that sexual size dimorphism in this group may have been selected for. Thus, small size may be favored in males because it allows greater mobility and because so much of their available energy is devoted to searching for females rather than to growth (Berry and Shine, 1980). I have found that male *M. leprosa* are smaller than females and that both their activity and mobility are higher than that of females during the mating season (unpublished data). The efficient location of sites that contain conspecific females

saves the males time and energy, and probably reduces exposure to predators during mate-searching (Magnhagen, 1991). In other groups, such as frogs or newts, chemical cues from conspecifics are used to assess whether potential mates are present (Wabnitz et al., 1999; Aragón et al., 2000).

Male *M. leprosa* preferred water from their home containers over clean water, and avoided water with chemical cues from other males during the mating season. This indicates that they were able to distinguish between their own odors and those of other males. Several studies have suggested that freshwater turtles have home ranges but are not territorial, and that home ranges of males show interindividual overlapping but that fighting between them is frequent (Lebboroni and Chelazzi, 1991; Kaufmann, 1992; Arvisais et al., 2002). High activity and mobility of males during the mating season and male–male competition for mates may increase the probability of interaction. The ability to discriminate and avoid chemical stimuli from other males, and to recognize their home area by their own odors may minimize agonistic encounters (see Gosling, 1990; Aragón et al., 2001).

The preference of females for water that contains chemical stimuli from other females during the mating season could be responsible for a grouping strategy used by them during reproduction. Gathering in certain areas would make it easier for males to find them, thus favoring the possibility of being fertilized by more than one male. Several studies show the existence of both multiple paternity and sperm competition in turtles (Galbraith et al., 1993; Valenzuela, 2000; Pearse et al., 2002). In evolutionary terms, it might be advantageous for female turtles to be fertilized by several males, promoting sperm competition (Galbraith, 1993). In this experiment, females responded actively to the odors of other females, but they did not to their own. This indicates that, similarly to the males, females were also able to discriminate between their own odors and those of other individuals of the same sex.

In aquatic organisms, there is no evidence that chemical cues are used to signal boundaries of territories. However, chemical communication could be used in close-range interactions. Turtles with the ability to recognize and locate chemical cues from conspecifics near them might have a selective advantage over turtles lacking such ability, because the ability to recognize and locate particular conspecifics without physical contact could be energetically advantageous. Thus, communication using conspecific chemicals may reduce time and energy costs for male and female *M. leprosa*. Since the reproductive interests are different for male and female turtles, and since conflicting strategies may have evolved between sexes (Berry and Shine, 1980; Galbraith, 1993), it is expected that they would show sex-specific responses to chemicals from other conspecifics in the mating season. Murphy et al. (2001) have suggested that sex-specific behaviors of *Sternotherus odoratus* could be due to sexual dimorphism in the transduction of chemosignals in the vomeronasal organ. In any case, the evolution of chemical communication in turtles and its implications for turtle behavior may have come from sexual

selection processes (see Rose, 1970; Eisner et al., 1977), like sexual size dimorphism (Berry and Shine 1980), and from other natural selection processes, such as for niche divergence (Lindeman and Sharkey, 1999). I suggest that communication using chemical cues may have a greater importance in turtle spatial orientation and sexual behavior than previously considered.

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PLANT VASCULAR ARCHITECTURE AND WITHIN-PLANT SPATIAL PATTERNS IN RESOURCE QUALITY FOLLOWING HERBIVORY

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Abstract—In this study, we used plant vascular architecture as a framework from which to predict induced changes in resource quality for *Lema trilinea* feeding on the host plant *Solanum dulcamara* at both low and high levels of herbivory. The systemic patterns of allocation of dye from a capillary tube inserted onto the petiole of the first true leaf and sections of the stem were used to establish the degree of vascular connectivity among different leaf positions. Induced changes in the activity of two defensive proteins, proteinase inhibitor (PI) and polyphenol oxidase (PPO), as well as larval *L. trilinea* performance, were measured in weakly or strongly connected leaves on plants with the first leaf damaged or undamaged by adult *L. trilinea*. At high levels of herbivory, larval performance decreased on the sixth leaf, which has strong vascular connections to the first leaf, yet increased on the fifth leaf, which has weak vascular connections to the first leaf. PPO activity increased in both the fifth and sixth leaf, while PI activity decreased in the fifth leaf although remaining unchanged in the sixth leaf. At low levels of herbivory, a decrease in larval performance was observed in the sixth leaf, but no change occurred in the weakly connected fifth leaf. Hence, plant vascular architecture clearly predicted within-plant changes in resource quality following only small amounts of herbivore damage.

Key Words—Induced responses, vascular architecture, *Solanum dulcamara*, systemic expression, orthostichy, *Lema trilinea*, leaf position, herbivory.

INTRODUCTION

Spatial heterogeneity in resource quality can affect the relative performance and preference of herbivores foraging on their host plants. For example, inter- or intraspecific variation in plant characteristics can influence herbivore growth,

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survival, fecundity, or dispersal (Denno and McLure, 1983; Bernays and Chapman, 1994), and potentially affect herbivore population dynamics (Denno and Peterson, 1995; Hunter and Price, 1995). In addition, *within-plant* resource heterogeneity can be an important determinant of herbivore foraging behavior and life history parameters (Jones et al., 1993; Suomela and Ayres, 1994; Barker et al., 1995).

Within-plant spatial heterogeneity has a number of causes. For example, tissues produced at different ontogenetic stages can be of different quality to herbivores: young leaves often have more nutrients, are softer, have more water, yet are more chemically defended than older plant parts (Raupp and Denno, 1983; Nichols-Orians and Shultz, 1990). In addition, environmental stimuli such as herbivore damage often induce chemical or morphological changes in plant phenotype (Karban and Baldwin, 1997), and may produce spatial heterogeneity when locally induced changes are stronger or different from systemic responses (Stout et al., 1996; Rojo et al., 1999). Another potentially important factor mediating within-plant heterogeneity in resource quality is plant vascular architecture.

Plant vascular architecture, which refers to the connectivity of vascular traces among different leaves on a plant, is central to functional models predicting within-plant spatial heterogeneity in resource quality (Orians and Jones, 2001). The vascular architecture of a plant can affect the movement of many compounds important to herbivore performance, including signal molecules, photosynthates, and hormones (e.g., Watson and Casper, 1984; Sprugel et al., 1991; Vuorisalo and Hutchings, 1996). Vascular architecture can also influence the movement of compounds induced by herbivory. Davis et al. (1991) showed that systemic expression of wound-induced mRNAs following mechanical damage to poplar leaves increased with vascular connectedness to the damaged leaf. A similar pattern has been found in tobacco for systemic acquired resistance (Shulaev et al., 1995) and the wound-induced expression of several defensive genes (Schittko and Baldwin, 2003), as well as for the induction of proteinase inhibitors (PIs) in tomato plants (Orians et al., 2000). Moreover, Jones et al. (1993) showed induced resistance against beetle larvae feeding on poplar leaves, but only if there was a high degree of connectedness to the site of damage.

However, severe wounding may reduce the constraining effects of vascular architecture on the movement of induced compounds. Jones et al. (1993) confined initial damage to a small area proximal to the midvein of a leaf, explicitly avoiding damage to 2, 3, and 4° traces that were known to connect nonorthostichous leaves (which are not in the same vertical axis and, therefore, do not share a main vascular trace). Rhoades et al. (1999) found that severity of mechanical wounding influenced the degree to which vascular architecture constrained systemic expression of PIs in tomato plants: small amounts of damage elicited systemic PI patterns consistent with strength of vascular connections between leaves, yet larger wounds induced more generally distributed PI activity.

In this study, we used the host plant *Solanum dulcamara* (bittersweet nightshade) and *Lema trilinea* (the three-lined potato beetle) (Coleoptera: Chrysomelidae) to examine whether plant vascular architecture can predict herbivore performance on different leaf positions following varying levels of damage by a leaf-feeding herbivore. Specifically, we addressed the following questions: (1) What is the relative strength of vascular connections between leaves in *S. dulcamara*? (2) How do larval *L. trilinea* perform on a leaf with strong connections to the first leaf, when reared on a plant where the first leaf is severely damaged versus undamaged? (3) How do larval *L. trilinea* perform on a leaf with weak connections to the first leaf, when the first leaf is severely damaged versus undamaged? (4) Are effects on herbivore performance in the two leaf positions similar at low levels of initial herbivory? (5) How does a high level of damage to the first leaf affect the expression of defensive proteins and carbon–nitrogen concentrations in the strongly and weakly connected leaf, respectively?

METHODS AND MATERIALS

Plant and Insect Rearing. L. trilinea adults were collected from the Koffler Scientific Reserve at Joker's Hill (hereafter Joker's Hill), King City, Ontario, and maintained on a 16:8 L:D cycle over multiple generations on *S. dulcamara* plants. For each experiment, *S. dulcamara* individuals were grown in ProMix DX soil contained in 131-ml pots from seeds collected at Joker's Hill. Plants experienced a 16:8 L:D cycle in a greenhouse for 3 wk until they had 8–10 true leaves, 6 of which were fully expanded.

Vascular Architecture of S. dulcamara. Two methods were used to assess the degree of connectivity between leaves in *S. dulcamara*. First, vascular architecture was determined through hand sections taken with a razor blade. Sectioning protocol is described in detail in O'Brien and McCully (1981). Cross sections obtained from each node and internode of two plants were stained with a 0.05% toluidine blue solution, wet-mounted on glass slides, and observed by using a Reichert–Jung polyvar microscope. Care was taken to preserve the alignment of sequential sections. Images were captured with a Nikon DXM 1200 digital camera mounted onto the microscope, and transferred into ACT-1 image analysis software for inspection. Vascular bundles stained blue were apparent in these images, and their position in sequential sections of the stem was used to infer the pattern of vascular connectedness between leaves.

The second protocol was similar to that used by Orians et al. (2000). The first true leaf on each of 10 plants was severed with a razor, and a capillary tube containing a 0.025% w/v solution of Rhodamine-B dye (Sigma, St. Louis, MO) was inserted onto the petiole. After 24 hr, the amount of dye in each fully expanded leaf was visually scored as low, medium, or high. The relative concentration of

dye taken up by each leaf reflects its degree of vascular connectivity with the first leaf (Orians et al., 2000). The effect of leaf position on dye score was analyzed by using a *G* test. In addition, two of the plants treated with dye were hand-sectioned and dry mounted on glass slides to determine if externally visible patterns of dye allocation matched dye movement through different traces in the plant.

Effects of High Levels of Herbivory and Connectivity Among Leaves on Larval Performance. To determine how differences in connectivity among leaves affect the performance of larvae on plants previously damaged by *L. trilinea* adults, a clip cage was placed on the first true leaf of *S. dulcamara* plants. Clip cages were made from the tops of ventilated Petri dishes (5 cm) attached to either side of a hair clip, and did not weigh down the leaf since they rested flat on the soil. One adult *L. trilinea* was placed in half of the cages. The beetles were allowed to feed for 24 hr, at which time approximately 60% of the first leaf had been consumed. All clip cages and herbivores were then removed. Twenty-four hours after herbivore removal a nascent larval *L. trilinea* was confined using Tanglefoot™ to one of two leaf positions on each plant: the fifth or the sixth leaf, which have weak and strong vascular connections, respectively, with the first true leaf (see Results, *The vascular architecture of* S. dulcamara). Larval mass was measured after 2 days of feeding on each independent leaf position/treatment combination (bioassays on the fifth and sixth leaf were performed on separate plants). This experiment was conducted twice, with 80 and 52 plants in each trial, respectively. Results were analyzed by a three-way ANOVA, with damage treatment, leaf position, and trial as the main effects. Significant differences were further examined using Tukey *post hoc* comparisons.

Effects of High Vs. Low Amounts of Herbivory and Connectivity Among Leaves on Larval Performance. To determine if level of initial herbivory influences whether connectivity among leaves can mediate induced changes in larval performance, 60 *S. dulcamara* plants were set up with three damage treatments: (1) two adult *L. trilinea* clipped onto the first leaf for 12 hr, resulting in approximately 45% of the leaf being eaten, (2) one adult *L. trilinea* clipped onto the first leaf for 12 hr, resulting in approximately 15% of the leaf being eaten, and (3) an empty clip cage placed on the first leaf for 12 hr. Bioassays with nascent larvae were subsequently conducted as described above (on the six independent leaf position/treatment combinations). Results were analyzed by a two-way ANOVA, with damage treatment and leaf position as the main effects. Significant differences were further examined using Tukey *post hoc* comparisons.

Effects of High Levels of Damage and Connectivity Among Leaves on Expression of Defensive Proteins. To test if high levels of herbivory by adult *L. trilinea* induced changes in the activity of defensive proteins in both leaf positions, in a separate experiment damage was imposed by using single beetles feeding for 24 hr, as described above. Twenty-four hours after herbivore removal, both the fifth and sixth leaf from each of 80 plants were harvested and immediately frozen.

Subsequently, these tissues were assayed for the activity of proteinase inhibitors (PIs) and polyphenol oxidases (PPO), which are putative defensive proteins in Solanaceous plants (e.g., Broadway et al., 1986; Felton et al., 1992; Thaler et al., 1996; Karban and Baldwin, 1997). PIs were measured in both leaf positions in 40 plants, PPO in the remaining half. Results for each compound were analyzed by using MANOVA, with damage treatment as the main effect and activity level in the fifth and sixth leaf of a given plant as response variables. A significant multivariate effect was followed by univariate comparisons.

PI activity was measured through the ability of plant extracts to inhibit the degradation of the peptide azocasein (Sigma) by the protein trypsin (Sigma) (Orians et al., 2000). By using a PowerGen 700 sonicator, 100 mg samples from each leaf were ground in pH 7.8–8.0 Tris HCl extraction buffer (3 μl/mg fresh weight) until homogenous. The ground samples were vortexed for 3 min, then centrifuged at 11,000 rpm for 10 min at 10°C. The supernatant was poured into standard microcentrifuge tubes. Sixty microliters of the supernatant was added to 20 μl of Tris buffer, 50 μl of 2% azocasein in Tris buffer, and 20 μl of a 0.001 M HCl solution containing 200 mg of trypsin. Following incubation for 20 min at 28°C, 100 μl of trichloroacetate (100% w/v) was added to stop the reaction. Samples were centrifuged for 10 min at 8000 rpm, and 100 μl of the supernatant for each sample was transferred into individual microplate wells (Corning Science Products, Corning, NY). One hundred microliters of NaOH was added to each well. Absorbance readings were taken at 450 nm with a microplate reader (PowerWave, Bio-Tek Instruments Inc., Winooski, VT). For every sample, a control was run with the substrate and trypsin only. Percent inhibition activities were calculated relative to this control and no-enzyme blanks (Orians et al., 2000). Higher PI activities were indicated by lower rates of change in absorbance.

PPO activity was measured by the methods described in Thaler et al. (1996). Briefly, enzymes were extracted from weighed leaflets that were homogenized in ice-cold buffer, and then centrifuged to obtain a clarified extract for enzyme analyses. The supernatant was added to a caffeic acid solution (2.92 mM in pH 8 K Phos buffer), and increases in optical density were measured at 470 nm.

Effects of High Levels of Damage and Connectivity Among Leaves on Carbon–Nitrogen Concentrations. To test if changes in carbon–nitrogen concentrations are induced by adult *L. trilinea* at high levels of damage, in a separate experiment damage was imposed by using single beetles feeding for 24 hr, as described above. Twenty-four hours after herbivore removal, tissue from the fifth and sixth leaf of 29 plants was harvested and assayed for total nitrogen and carbon content (% mass per dry gram of leaf) by using a LECO, CHN Analyser (St. Joseph, MO). Results for each compound were analyzed with MANOVA, with damage treatment as the main effect and activity level in the fifth and sixth leaf of a given plant as response variables.

RESULTS

Vascular Architecture of S. dulcamara. The systemic pattern of dye allocation indicated vascular differentiation in *S. dulcamara*: the amount of dye found in each leaf was affected by its position on the stem ($G = 95.02$, $P < 0.001$) (Figure 1a). The sixth leaf, which is orthostichous with the first leaf (i.e., in the same vertical

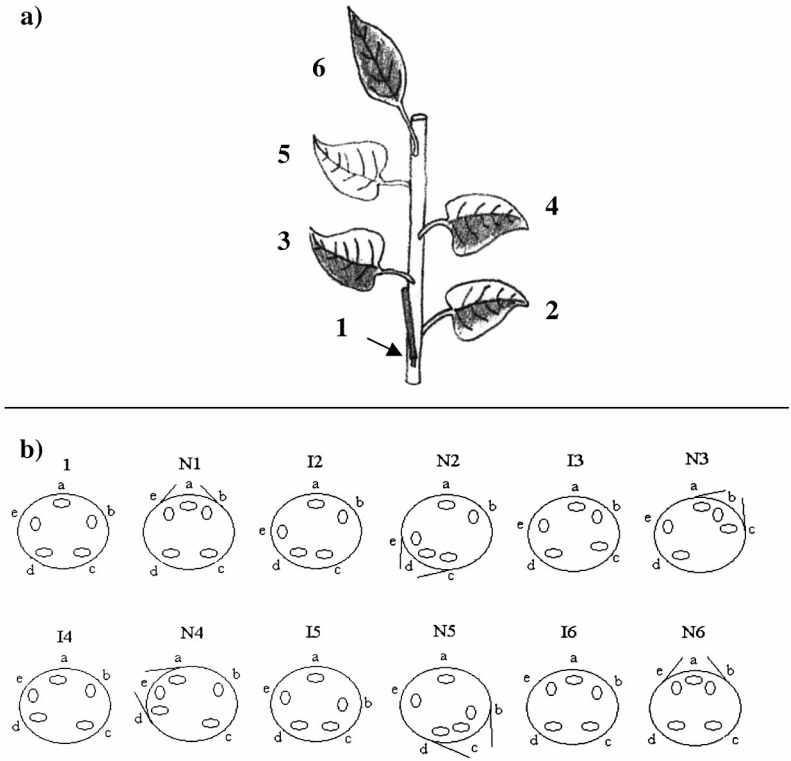

FIG. 1. (a) Drawing of *Solanum dulcamara* showing systemic uptake of dye fed through the cut petiole of leaf 1 using a capillary tube. Leaf positions are numbered in ascending order from the first to the sixth true leaf. Leaf positions 1 and 6 are orthostichous (in the same vertical axis). (b) Cross sections of *S. dulcamara* stem, showing each internode (I) and node (N) in ascending order through leaf positions 1–6. The five ovals in each section represent vascular traces, labeled a–e. Petioles shown at each node are provisioned by one median and two lateral traces. Moving in a counterclockwise direction, a given lateral trace at each node twists around the stem to become one of the lateral traces for the petiole in the node above it (e.g., trace e in nodes 1 and 2). Following this pattern, orthostichous leaves (1 and 6) share the exact same median and lateral traces, while any two leaves have at least one trace in common.

axis), was fully flushed with red dye in 10 out of 10 plants. In contrast, the fifth leaf, which is in the opposite vertical axis from the first leaf, did not visibly change in color in any of the plants. In the second, third, and fourth leaves, dye was present only on one side of the midvein, in the half of each leaf proximal to the vertical axis of the first leaf (Figure 1a).

Hand sections of the stem confirmed that orthostichous leaves share strong vascular connections. Each leaf is provisioned by three vascular traces, and for the first and sixth leaves the median and lateral traces are exactly the same (Figure 1b). All leaves have some degree of vascular connectivity, however, as any given pair of leaves share at least one vascular trace. The first and fifth leaves are connected by a common lateral trace (trace b in Figure 1b).

Hand sections of the stem and petioles of dyed plants indicated that systemic movement of the dye occurred primarily through the three traces provisioning the first leaf (traces a, b, and e in Figure 1b). Thus, the pattern of dye allocation to the third, fourth, and sixth leaves is explained by the number and position of traces they share with the first leaf: the more traces in common with leaf position one, the greater the amount of dye accumulation (compare Figure 1a and b). Consistent with a low degree of connectivity, no dye was detectable in the fifth leaf or its sectioned petiole, although dye was present in the lateral trace outside this leaf at the fifth node. Conversely, dye accumulated in the second leaf, possibly because of its proximity to the first leaf. In addition, some lateral movement of dye into all traces was observed in the second and third internodes because of the presence of vascular cambium. Dye in traces not connected to the first leaf may accumulate in the second leaf after diffusing through the vascular cambium, but decrease to undetectable concentrations by the time it reaches the fifth leaf.

Effects of High Levels of Damage and Connectivity Among Leaves on Larval Performance. Leaf position affected larval performance differently in the damaged and undamaged treatments (treatment: $df = 1$, $F < 0.01$, $P = 0.97$; leaf: $df = 1$, $F = 0.48$, $P = 0.49$; Treatment $\times$ Leaf interaction: $df = 1$, $F = 28.2$, $P < 0.01$). The sixth and fifth leaves differ in constitutive resource quality for larval *L. trilinea*, indicated by the 19% greater mass of larvae reared on the sixth compared to the fifth leaf of undamaged plants (Figure 2). However, performance of larvae on damaged plants was reversed: larval mass was 24% lower on the sixth vs. the fifth leaf (Figure 2). The reversal resulted from larvae growing 24% less on the sixth leaf when the first leaf had previously been damaged relative to when it was undamaged (induced resistance), yet growing 21% more on the fifth leaf following damage to the first leaf (induced susceptibility) (Figure 2).

Effects of High Vs. Low Amounts of Herbivory and Connectivity Among Leaves on Larval Performance. Leaf position interacted with damage treatment to influence larval performance (treatment: $df = 2$, $F = 3.8$, $P = 0.028$; leaf: $df = 1$, $F = 4.46$, $P = 0.039$; Treatment $\times$ Leaf interaction: $df = 2$, $F = 21.1$, $P < 0.001$). On the sixth leaf, low levels of herbivory were sufficient to produce the

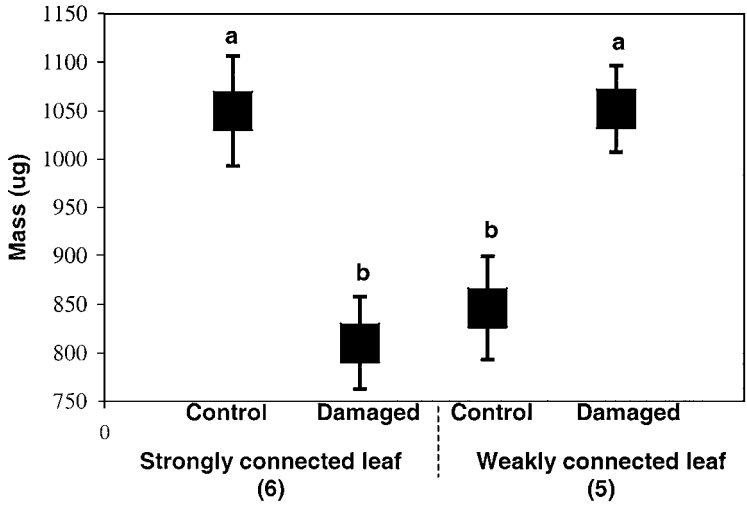

FIG. 2. Mass of *Lema trilinea* larvae after 2 days of feeding on *Solanum dulcamara* plants where the first true leaf was damaged (~60% of first leaf eaten) or undamaged by adult *L. trilinea*. Larvae were confined either to the sixth or to the fifth leaf. Boxes and bars give means ± SE, and different letters represent statistically significant Tukey *post hoc* comparisons.

decline in herbivore performance observed at higher levels of damage (Figure 3). However, larval performance on the fifth leaf did not change after a low amount of damage to the first leaf, but only following more severe wounding (Figure 3).

Effects of High Levels of Damage and Connectivity Among Leaves on Expression of Defensive Proteins. PI activity did not change in the sixth leaf following a high level of damage to the first leaf ($df = 1, 34$, $F < 0.001$, $P = 0.99$), yet declined by 70% in the fifth leaf ($df = 1, 34$, $F = 15.1$, $P < 0.001$) (Figure 4). Hence, PI activity does not correlate with induced resistance in the sixth leaf, yet is consistent with induced susceptibility in the fifth leaf. PPO activity increased in both leaf positions (leaf 5: $df = 1, 36$, $F = 4.14$, $P = 0.049$; leaf 6: $df = 1, 36$, $F = 4.1$, $P = 0.05$) (Figure 5), which correlates with induced resistance in the sixth leaf, but is inconsistent with induced susceptibility in the fifth leaf.

Effects of High Levels of Damage and Connectivity Among Leaves on Carbon–Nitrogen Concentrations. Carbon–nitrogen concentrations (% per dry gram of leaf) did not change in the assay leaves following damage (MANOVA: carbon: $df = 2, 26$, $F = 0.44$, $P = 0.65$; nitrogen: $df = 2, 26$, $F = 1.37$, $P = 0.27$). In the fifth leaf of undamaged and damaged plants, carbon concentrations were 41.36 ± 0.4 and 40.78 ± 0.39, while nitrogen concentrations were 5.67 ± 0.17

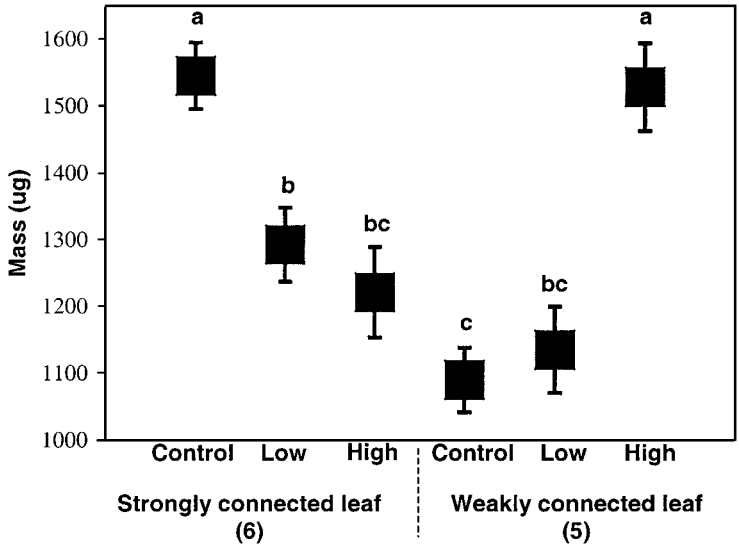

FIG. 3. Mass of *Lema trilinea* larvae after 2 days of feeding on *Solanum dulcamara* plants where the first true leaf was either undamaged or damaged by adult *L. trilinea*. Two levels of damage were imposed: low (~15% of first leaf eaten) or high (~45% of first leaf eaten). Larvae were confined either to the sixth or to the fifth leaf. Boxes and bars give means ± SE, and different letters represent statistically significant Tukey *post hoc* comparisons.

and 5.75 ± 0.11, respectively. In the sixth leaf of damaged and undamaged plants, carbon values were 41.84 ± 0.40 and 41.81 ± 0.51, while nitrogen values were 5.81 ± 0.18 and 6.10 ± 0.11.

DISCUSSION

A number of studies have found that leaf connectivity can mediate the expression of defensive compounds following several methods of induction: mechanical damage (Davis et al., 1991; Rhoades et al., 1999; Orians et al., 2000), pathogen infection (Shulaev et al., 1995), and application of herbivore saliva to mechanical wounds (Schittko and Baldwin, 2003). Only one previous study has matched plant vascular architecture with induced changes in herbivore performance (Jones et al., 1993). While each of these studies has shown that induced changes do not occur in leaves weakly connected to the site of damage (but see Rhoades et al., 1999), we find that high levels of tissue removal by a leaf-feeding herbivore can produce significant changes in the concentration of defensive compounds and herbivore performance in both strongly and weakly connected leaf positions.

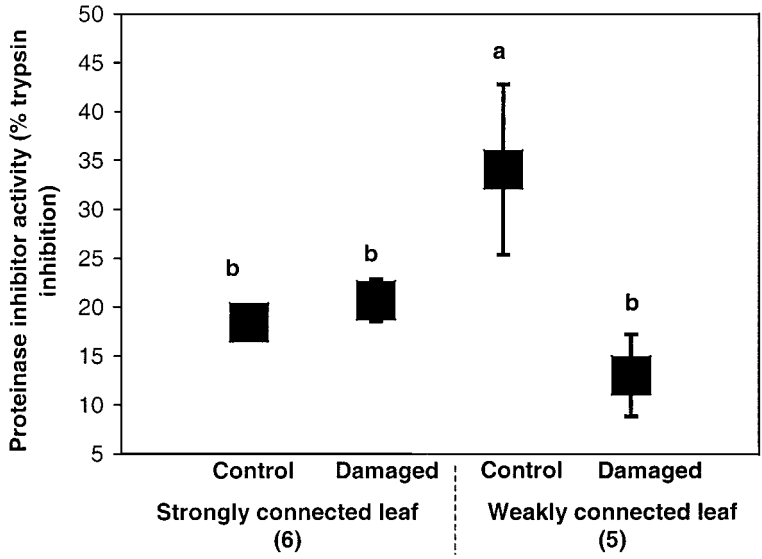

FIG. 4. Proteinase inhibitor activity in the sixth and fifth leaf of *Solanum dulcamara* plants where the first true leaf was damaged (~60% of first leaf eaten) or undamaged by adult *Lema trilinea*. Boxes and bars give means ± SE, and different letters represent statistically significant Tukey *post hoc* comparisons.

Independent of whether the measured defensive proteins are responsible for induced changes in *L. trilinea* larval mass, the performance of herbivores feeding on either the fifth or sixth leaves was significantly affected by high levels of damage to the plant. The most striking difference was not the magnitude of this effect, but its direction: induced resistance was observed in the strongly connected leaf and induced susceptibility in the weakly connected leaf. At low levels of herbivory, conversely, predictions based on vascular architecture were met: herbivore performance was altered following damage in the strongly, but not the weakly, connected leaf (Figure 3).

This pattern of change in herbivore performance on the fifth leaf suggests that compounds moving from the first leaf reach the fifth leaf only at high levels of herbivory. Functional differences in the strength of connections among different leaves indicated by the systemic pattern of dye fed through the first true leaf of *S. dulcamara* did not restrict changes in the activity of defensive proteins across leaf positions at high levels of damage. PI activity did not change in the sixth leaf, though it decreased significantly in the fifth leaf (Figure 4), while PPO activity increased significantly in both leaf positions (Figure 5). Communication between weakly connected leaves may have resulted from a greater quantity of induced

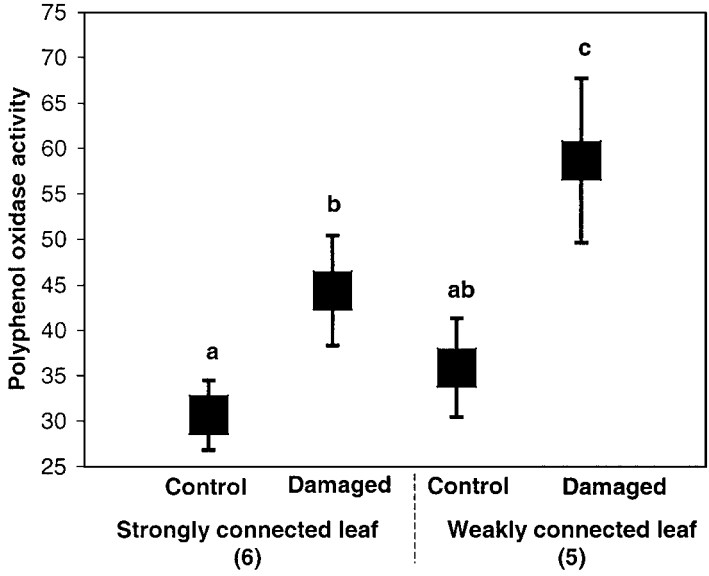

FIG. 5. Polyphenol oxidase activity in the sixth and fifth leaf of *Solanum dulcamara* plants where the first true leaf was damaged (~60% first leaf eaten) or undamaged by adult *Lema trilinea*. Boxes and bars give means ± SE, and different letters represent statistically significant Tukey *post hoc* comparisons.

compounds or signals being produced following severe herbivory. For example, Zwieniecki et al. (2003) found that hydraulic transport between vascular sectors in tomato plants is facilitated by high ion concentrations in the xylem. Additionally, enhanced uptake of induced compounds following severe damage to leaf veins that provision the lateral trace connecting the first and fifth leaf (see Figure 1b) may have contributed to the observed changes in defensive protein activity.

When examining specific induced responses, the decline in PI activity in only the fifth leaf at high levels of damage is puzzling. This result is not easily explained by a simple reduction in the constraining effect of vascular architecture on the movement of induced compounds, pointing instead to differences in physiology among leaf positions that are not determined by vascular connectedness to the site of damage. Such differences may additionally have resulted in induced susceptibility in this study, if decreased PI activity was responsible for increased *L. trilinea* performance on the fifth leaf. When examining primary compounds, carbon–nitrogen concentrations were not found to change in either leaf position following high levels of damage and were similar constitutively, suggesting no interplay between foliar nutrients and secondary compounds in affecting herbivore performance.

Overall, an explicit consideration of plant vascular architecture is undoubtedly essential for any attempt to predict within-plant spatial heterogeneity in resource quality for herbivores (Orians and Jones, 2001). Starting from this premise, we found that leaf position affected systemic alterations in resource quality for the herbivore *L. trilinea* in the host plant *S. dulcamara*, but degree of vascular connectivity among leaves predicted the magnitude of these effects only at low levels of herbivory. A greater amount of damage produced strong induced responses across different leaf positions, indicating that severity of herbivore wounding in concert with plant vascular architecture may greatly influence changes in resource quality following herbivore attack.

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DENSITY-DEPENDENT REDUCTION AND INDUCTION OF MILKWEED CARDENOLIDES BY A SUCKING INSECT HERBIVORE

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Abstract—The effect of aphid population size on host-plant chemical defense expression and the effect of plant defense on aphid population dynamics were investigated in a milkweed-specialist herbivore system. Density effects of the aposematic oleander aphid, *Aphis nerii,* on cardenolide expression were measured in two milkweed species, *Asclepias curassavica* and *A. incarnata*. These plants vary in constitutive chemical investment with high mean cardenolide concentration in *A. curassavica* and low to zero in *A. incarnata.* The second objective was to determine whether cardenolide expression in these two host plants impacts mean *A. nerii* colony biomass (mg) and density. Cardenolide concentration (μg/g) of *A. curassavica* in both aphid-treated leaves and opposite, herbivore-free leaves decreased initially in comparison with aphid-free controls, and then increased significantly with *A. nerii* density. Thus, *A. curassavica* responds to aphid herbivory initially with density-dependent phytochemical reduction, followed by induction of cardenolides to concentrations above aphid-free controls. In addition, mean cardenolide concentration of aphid-treated leaves was significantly higher than that of opposite, herbivore-free leaves. Therefore, *A. curassavica* induction is strongest in herbivore-damaged tissue. Conversely, *A. incarnata* exhibited no such chemical response to aphid herbivory. Furthermore, neither host plant responded chemically to herbivore feeding duration time (days) or to the interaction between herbivore initial density and feeding duration time. There were also no significant differences in mean colony biomass or population density of *A. nerii* reared on high cardenolide (*A. curassavica*) and low cardenolide (*A. incarnata*) hosts.

Key Words—*Aphis nerii*, *Asclepias,* cardenolide, chemical defense, herbivory, induction, milkweed, reduction, sequestration, specialist, trophic interactions.

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INTRODUCTION

Many plant families utilize rapidly mobilized, damage-induced chemical defense systems in response to insect herbivory. Phytochemical induction in response to mandibulate insect herbivory has been widely demonstrated where chewing insects cause conspicuous plant tissue damage and foliar biomass reduction (Karban and Baldwin, 1997). However, little is known about the defensive response of plants to haustellate insect herbivory, where photosynthate is typically sucked from phloem vasculature, causing little plant tissue damage or foliar biomass loss. Nevertheless, haustellate herbivory generates a sink for the products of primary metabolism that increases as individual herbivores grow and their local populations increase in size.

Malcolm and Zalucki (1996) found that common milkweed *Asclepias syriaca* (Apocynaceae, Asclepiadoideae) induces chemical defense expression in response to mechanical leaf damage like that caused by mandibulate chewing of herbivores such as larvae of the monarch butterfly, *Danaus plexippus.* The oleander aphid, *Aphis nerii* B. de F., is another widely distributed milkweed herbivore that, in contrast to monarch larvae, does not cause conspicuous host tissue damage because it feeds by inserting haustellate maxillary stylets directly into phloem tissue (Botha et al., 1977; Malcolm, 1981). Nothing is known about the effect that *A. nerii* feeding has on milkweed chemical defense expression, or the effect of variable defense expression on aphid fitness correlates.

A. nerii is a bright yellow, aposematic aphid with a worldwide distribution that is restricted to feeding on plants in the Apocynaceae, especially the subfamily Asclepiadoideae (Patch, 1938; Ismail and Swailem, 1971; Price and Willson, 1979; Malcolm, 1991). This common aphid is found wherever milkweeds and oleander occur and it is known to sequester cardenolides, phloem-mobile host allelochemicals, for use as a chemical defense against natural enemy attack (Rothschild et al., 1970; Pasteels, 1978; Duffey, 1980; Malcolm, 1981, 1986, 1989, 1990, 1992; Dixon, 1998). Cardenolides are potent, steroidal Na^+/K^+-ATPase inhibitors that are toxic to most consumers and are characteristic defenses of plants in the Apocynaceae (Malcolm, 1991). *A. nerii* feed in the cardenolide-rich internal phloem of host bicollateral vascular bundles (Botha et al., 1977; Malcolm, 1981), and these chemicals are present in *A. nerii* honeydew and body tissue (Bailey, 1974; Malcolm, 1990).

We investigated the impact of *A. nerii* colony density and feeding duration on host cardenolide expression, and the effect of host cardenolide concentration on *A. nerii* colony size and biomass. Our first objective was to determine whether *A. nerii* feeding influences cardenolide expression in two milkweed species that vary in constitutive chemical defense investment. *Asclepias curassavica* L. has high constitutive leaf cardenolide content, while *A. incarnata* L., the swamp milkweed, has low constitutive leaf cardenolide content (Roeske et al., 1976; Malcolm, 1990,

1991). Our second objective was to determine whether cardenolide expression in these two host plants differentially impacts *A. nerii* colony size and biomass.

METHODS AND MATERIALS

Plant Culture. Ninety North American *A. incarnata* were purchased from a grower in Kalamazoo, Michigan, USA (Van Bochove's Greenhouse Direct: plants grown from Michigan-collected seeds), and 90 neotropical *A. curassavica* were pot-reared from seed at the Western Michigan University greenhouse complex (seeds originated from greenhouse plants grown at Michigan State University). All plants were approximately 4 months old at the initiation of the experiment, were grown in MetroMix^{TM} 410 soilless medium, and were maintained insect-free in a sealed greenhouse room until application of *A. nerii* in clip cages in the field (no pesticides were used on these plants). In addition to the marked difference in constitutive cardenolide content, the two species were chosen for this experiment because their leaf morphologies and growth forms are similar. Both species are erect perennials with smooth, entire, lanceolate leaves of equivalent leaf area (Woodson, 1954). Woodson (1954) describes *A. curassavica* as a neotropical annual; however, greenhouse-grown plants do perennate for several years.

Phytochemical Analysis. Cardenolides were extracted from harvested leaf samples by solid phase extraction and analyzed by high performance liquid chromatography (HPLC) using the methods of Wiegrebe and Wichtl (1993) as modified by Malcolm and Zalucki (1996). Mean foliar cardenolide concentrations for herbivore-free control plants harvested throughout the experiment were *A. curassavica* $= 1421$ μg/g (SEM $= 216$ μg/g, $N = 15$) and *A. incarnata* $= 0$ μg/g ($N = 15$).

Insect Culture. Aphid cultures were established from natural populations feeding on *A. syriaca* and *A. incarnata* in Kalamazoo County, Michigan, USA. Prior to experimentation, aphids to be used on *A. curassavica* were maintained on *A. curassavica*, while aphids to be used on *A. incarnata* were maintained on *A. incarnata* for at least five generations.To start the experiment, fifth instar, apterous *A. nerii* were placed in one of six logarithmically increasing starting densities (0, 3, 7, 20, 55, or 148), onto a single leaf of the third leaf pair from the plant apex. Each plant contained a single aphid colony confined to this location. The insects were enclosed in foam-backed PVC (polyvinylchloride) clip cages. The 4-cm diam clip cages were made from two 3-mm sections of PVC pipe held on opposite sides of a leaf by a spring hair clip. Nylon sheer mesh (0.1 mm^2) was glued on the outside of each PVC section to form an enclosure. In an effort to limit the potential for clip-cage-induced leaf tissue damage, only the foam made direct contact with the leaf surface. Clip cages were supported by twist-ties attached to 3-ft support stakes used to anchor each potted plant. All insects were allowed to reproduce and feed

freely within the confines of their respective enclosures until harvest. Aphids were confined to the lower, abaxial leaf surface, so excreted honeydew did not contact the surface of leaves analyzed for cardenolide content.

Experimental Procedure. The six insect starting density treatments (0 [control], 3, 7, 20, 55, 148) reflect a range of single leaf colony densities commonly found in nature (Ismail and Swailem, 1971; Malcolm, 1976; Hall and Ehler, 1980; Groeters, 1989; Groeters and Dingle, 1989; Bristow, 1991). The experimental design included 5 sampling days $\times$ 6 initial herbivore treatment densities $\times$ 2 milkweed species $\times$ 3 replicates/plant species $=$ 180 plants (90 of each species). The two groups of 90 plants were each divided into three, 30 plant replicate groups/plant species as illustrated (Table 1).

All greenhouse-grown plants were maintained outside under ambient conditions for 6 days (June 10–15) prior to the initiation of the experiment (June 16). The assay was conducted in a flat, 240 m^2 area of mowed lawn divided equally into six units (3 replicates $\times$ 2 plant species) (Table 1). The day before the experiment began, all plants were arranged in conspecific replicate units 1 m from each nearest neighbor. Each replicate contained 25 treatment (+aphids) and 5 control plants (−aphids). Plants in a given replicate were arranged into rows, each containing six plants. Therefore, all rows were composed of plants representing the full complement of aphid starting densities (Table 1). The particular sequence of aphid starting density treatments in each row was predetermined to limit the potential for interactions between colony density and plant position.

To assess both the effects of aphid starting density and feeding duration time on cardenolide expression, one row was removed/replicate/plant species every 24 hr for 5 days. Two leaf samples were harvested from each plant (third leaf pair) to test whether phytochemical responses were systemic or localized to aphid-damaged tissue ($N = 360$). At the time of leaf harvest, all leaves were weighed wet. Samples were then lyophilized and reweighed, and cardenolides were extracted using the phytochemical analysis procedure described in Malcolm and Zalucki (1996). Aphids were counted according to three instar groups (first + second, third + fourth, and fifth alates + apterae) and then weighed collectively in grouped instars. These weights were then summed to give the total aphid biomass.

Statistical Analyses. Collected data were analyzed with both StatView™, SuperAnova™, and JMP™ v5 software (SAS Institute, Cary, NC) and tested for normality (Shapiro–Wilk W) and homogeneity of variances (O'Brien, Brown–Forsythe, Levene, or Bartlett tests) followed by ANOVA, ANCOVA, and regression analyses. Most of the 480 measures of aphid (aphid N, Ln aphid $N + 1$, aphid biomass) and plant response (leaf cardenolide content) were normally distributed among treatments and replicates, with the exception of 19 measures (4%): these included aphid measures at density 7 on *A. incarnata* on days 1 and 2, density 148 on *A. incarnata* on day 1, density 20 on *A. curassavica* and *A. incarnata* on day 3, and density 3 on *A. curassavica* on day 4. These data were excluded

TABLE 1. EXPERIMENTAL DESIGN AND ORIENTATION OF BOTH HOST PLANT AND 6 APHID DENSITY TREATMENTS (0 = CONTROL, 3, 7, 20, 55, 148) DISTRIBUTED AMONG 3 REPLICATES

Host plant	Replicate 1						Replicate 2						Replicate 3					
A. incarnata	3	7	20	55	148	C	3	7	20	55	148	C	3	7	20	55	148	C
	C	148	3	7	20	55	C	148	3	7	20	55	C	148	3	7	20	55
	55	7	C	148	3	20	55	7	C	148	3	20	55	7	C	148	3	20
	148	3	20	7	C	55	148	3	20	7	C	55	148	3	20	7	C	55
	7	C	55	20	3	148	7	C	55	20	3	148	7	C	55	20	3	148
A. curassavica	3	7	20	55	148	C	3	7	20	55	148	C	3	7	20	55	148	C
	C	148	3	7	20	55	C	148	3	7	20	55	C	148	3	7	20	55
N⇑	55	7	C	148	3	20	55	7	C	148	3	20	55	7	C	148	3	20
	148	3	20	7	C	55	148	3	20	7	C	55	148	3	20	7	C	55
	7	C	55	20	3	148	7	C	55	20	3	148	7	C	55	20	3	148

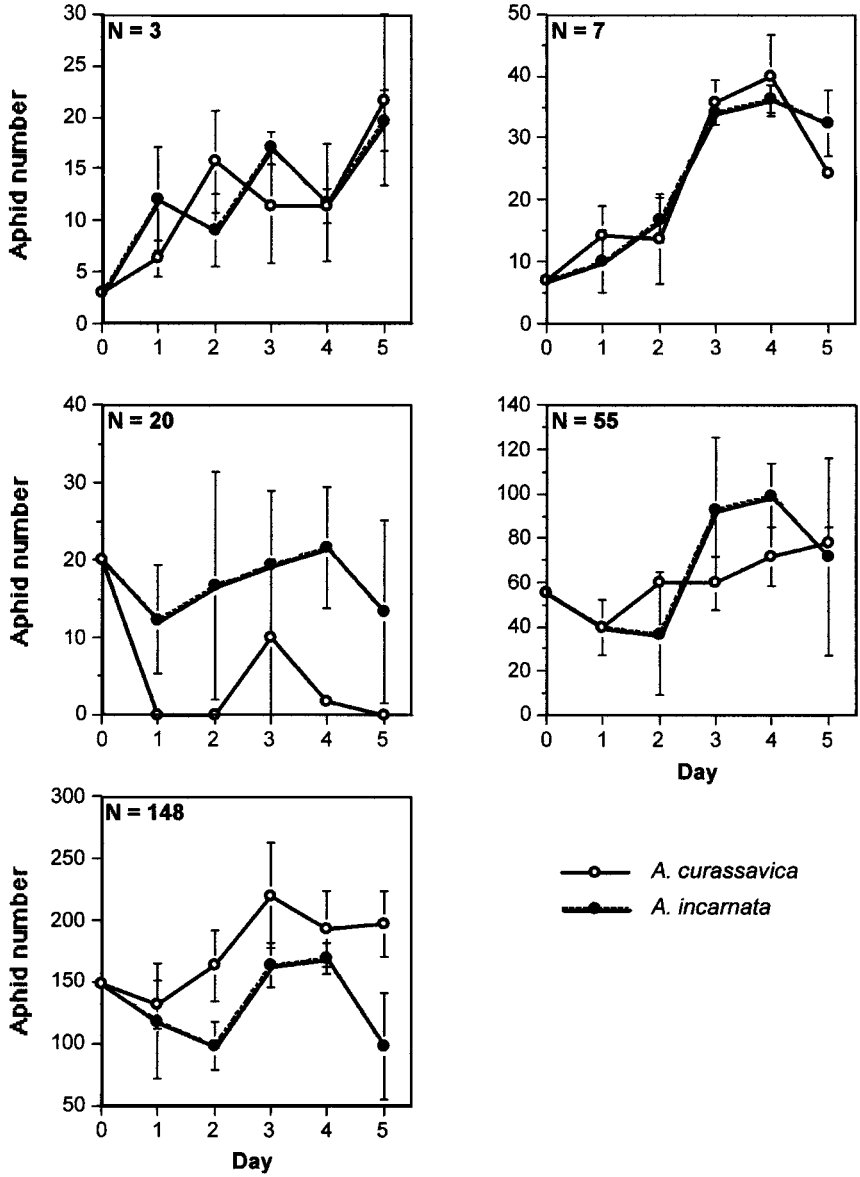

FIG. 1. Mean aphid numbers (±SEM) plotted against time for each initial density treatment on *A. curassavica* and *A. incarnata.*

from subsequent analyses because transformation made little difference to their distributions. In ANOVA comparisons of aphid performance between the two host plants, or plant cardenolide responses to aphid densities across days, all variances were homogeneous for one or more tests of homogeneity.

RESULTS

Aphid Population Dynamics. In each treatment, aphid populations increased significantly with time, except for the 20 density treatment on both host plants and the 55 and 148 density treatments on *A. incarnata* (Figure 1, Table 2). Density treatment 3 showed an overall increase, while density 7 growth on both host plants suggested sigmoidal increase to a possible asymptote after 4 days. This asymptote might result from interference competition for space within the clip cages. Such competition may also have limited the higher density treatments to less (N = 20) and more variable increase (N = 55, 148) in aphid density with time (Figure 1).

Overall, total aphid biomass on each host plant increased significantly and linearly with aphid numbers [*A. curassavica* aphid biomass (mg) = 0.46 aphid N, $r^2 = 0.80$, $F_{(1,\ 72)} = 289.4$, $P < 0.001$; *A. incarnata* aphid biomass (mg) = 0.50 aphid N, $r^2 = 0.73$, $F_{1,84} = 228.5$, $P < 0.001$], and there was no significant difference between the slopes of these linear regressions constrained through the origin [ANCOVA, $F_{1,155} = 0.19$, ns]. Thus, aphid numbers were used in the analysis of aphid dynamics rather than aphid biomass. In the case of the *A. curassavica* 20 treatment, we collected insufficient data because the aphid populations crashed in all three replicates for unknown reasons. Consequently, we are unable to compare these data with those from *A. incarnata.*

TABLE 2. LINEAR REGRESSIONS OF APHID NUMBERS AGAINST TIME IN DAYS FOR EACH INITIAL APHID DENSITY ON *A. curassavica* AND *A. incarnata* AND ANALYSES OF COVARIANCE OF REGRESSION SLOPES BETWEEN THE TWO HOST PLANTS

		Linear regression				ANCOVA	
Host	Density	Regression	r^2	$F_{(1,\ 17)}$	P	Host $F_{(1,\ 31)}$	P
A. curassavica	3	$N = 4.11 + 2.98$ d	0.32	7.68	0.014	0.05	ns
A. incarnata	3	$N = 5.05 + 2.71$ d	0.41	10.20	0.006		
A. curassavica	7	$N = 9.21 + 5.31$ d	0.43	12.28	0.003	0.01	ns
A. incarnata	7	$N = 8.62 + 5.96$ d	0.73	40.08	<0.001		
A. curassavica	20	—	—	—	—	—	—
A. incarnata	20	$N = 21.13 + 0.42$ d	0.004	0.04	ns		
A. curassavica	55	$N = 45.57 + 6.03$ d	0.32	7.49	0.015	0.02	ns
A. incarnata	55	$N = 43.19 + 9.10$ d	0.13	2.30	ns		
A. curassavica	148	$N = 140.71 + 13.93$ d	0.23	4.70	0.046	0.04	ns
A. incarnata	148	$N = 134.64 - 0.77$ d	0.001	0.01	ns		

TABLE 3. TWO-WAY ANOVA OF THE EFFECTS OF *A. nerii* FEEDING DURATION TIME (DAYS), INITIAL *A. nerii* DENSITY, AND INTERACTION, ON THE CARDENOLIDE CONCENTRATIONS (μg/g LEAF DRY WEIGHT) OF *A. curassavica* AND *A. incarnata*

A. curassavica (μg/g)[a]						*A. incarnata* (μg/g)[a]					
Density	Mean	SE	Day	Mean	SE	Density	Mean	SE	Day	Mean	SE
0	1419	216	1	1401	1102	0	0	0	1	0	0
3	661	110	2	1120	911	3	7.6	7.6	2	7.6	7.6
7	685	95	3	959	675	7	23.6	17.3	3	4.6	4.6
20	563	113	4	1328	880	20	46.0	25.9	4	40.7	22.3
55	1332	129	5	1248	1358	55	14.4	10.6	5	23.5	14.7
148	2608	336				148	0	0			

		A. curassavica		*A. incarnata*	
Source	df	*F*	*P*	*F*	*P*
Time (d)	4	1.04	ns	1.84	ns
A. nerii treatment density	5	16.90	<0.001	1.68	ns
Time $\times$ Density interaction	20	0.92	ns	1.01	ns
Residual	60				

[a] Cardenolide concentrations by day ($N = 18$ each day) and density ($N = 15$ for each density).

When slopes of linear regressions of aphid numbers against time for each aphid density treatment were compared between *A. curassavica* and *A. incarnata* using analysis of covariance (ANCOVA), we found no significant differences (Table 2). Thus, host-plant species had no effect on the rates of increase of aphid populations for all starting densities.

Leaf Cardenolide Content. There was a significant effect of aphid density on the cardenolide content of *A. curassavica* leaves, but not *A. incarnata* leaves (Table 3). However, there was no time effect of aphid feeding duration on the cardenolide content of leaves for either host plant (Table 3). In addition, there were no significant Feeding Duration $\times$ Aphid Density interactions (Table 3). Thus, the cardenolide responses of leaves that supported each aphid density, and leaves opposite these aphid colonies, showed little change through time (Figures 2 and 3). Both clip cage leaves with the highest initial aphid density of 148, and leaves opposite these colonies on *A. curassavica* showed a decrease in cardenolide content on days 2 and 3. This was followed by an increase back to the original cardenolide concentrations. Control plants without aphids all showed cardenolide concentrations that were higher than all aphid density treatments, except the highest initial density of 148 aphids (Figure 2).

No regressions of cardenolide content against time were significant (Table 4) for clip cage and opposite leaves from *A. curassavica.* This reaffirmed the lack of influence of feeding duration in the two-way ANOVA (Table 3). There was so

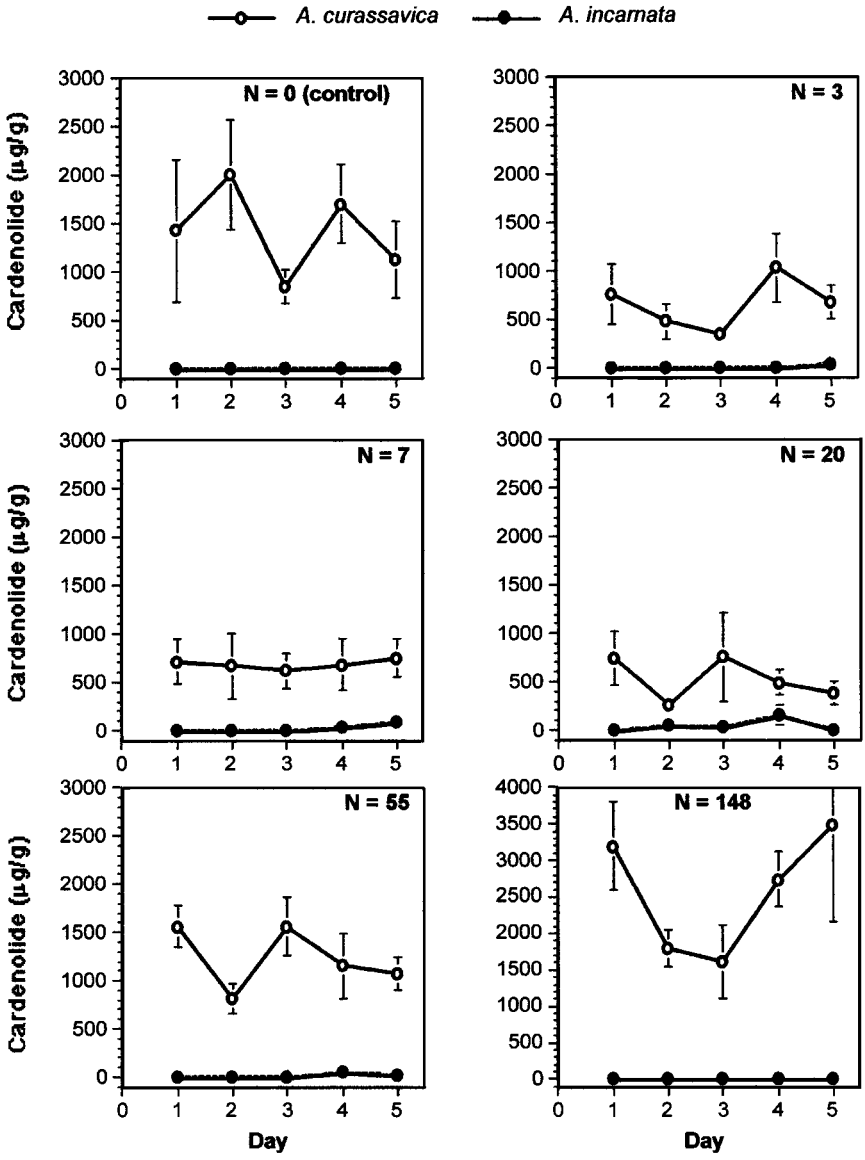

FIG. 2. Change in mean cardenolide concentrations in aphid leaves of *A. curassavica* and *A. incarnata* with time.

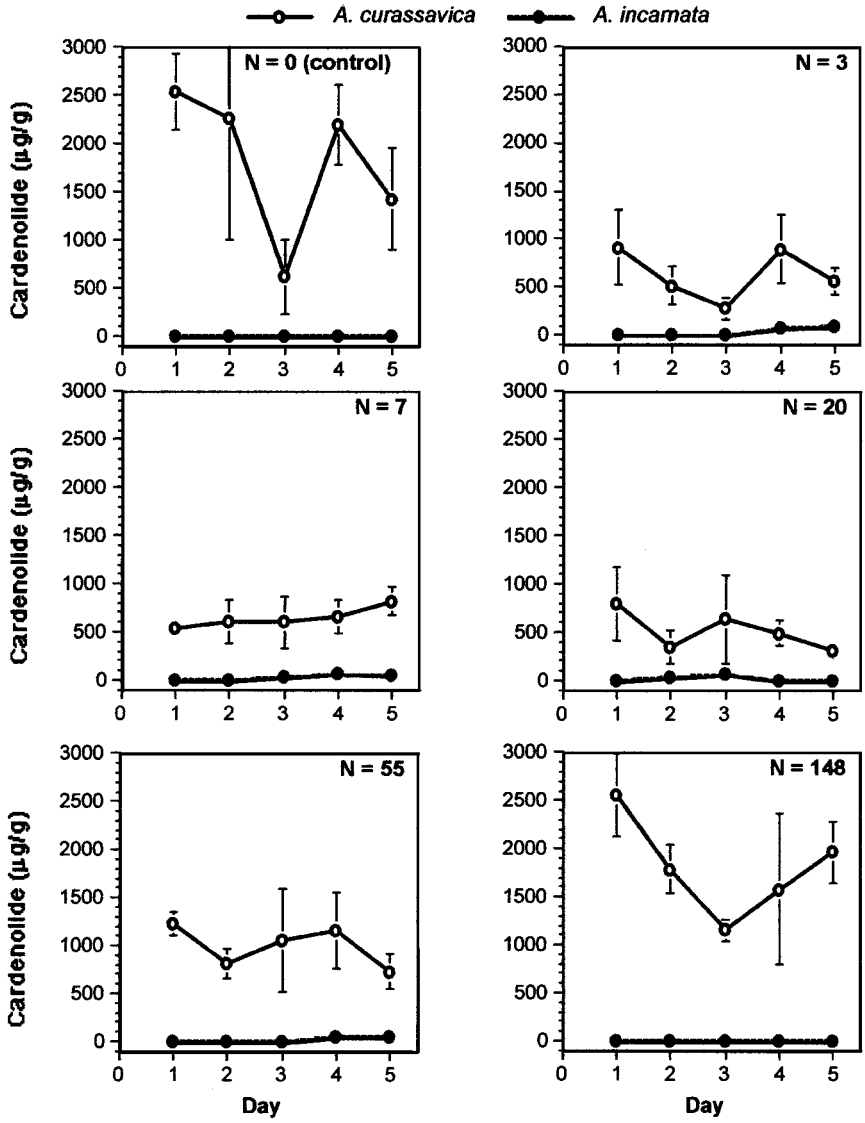

FIG. 3. Change in mean cardenolide concentrations in opposite leaves of *A. curassavica* and *A. incarnata* with time.

TABLE 4. LINEAR REGRESSIONS OF CARDENOLIDE CONCENTRATIONS (μg/g LEAF DRY WEIGHT) IN APHID LEAVES (FIGURE 2) AND OPPOSITE LEAVES (FIGURE 3) AGAINST TIME (d)

Host	Density	Leaf	Regression	r^2	$F_{(1, 14)}$	P
A. curassavica	0	aphid	$\mu g = 1697.2 - 92.6$ d	0.03	0.35	ns
A. curassavica	0	opposite	$\mu g = 2504.4 - 232.0$ d	0.08	1.06	ns
A. curassavica	3	aphid	$\mu g = 547.5 + 37.9$ d	0.02	0.22	ns
A. curassavica	3	opposite	$\mu g = 722.1 - 30.6$ d	0.009	0.12	ns
A. curassavica	7	aphid	$\mu g = 663.2 + 7.1$ d	0.001	0.92	ns
A. curassavica	7	opposite	$\mu g = 462.8 + 60.6$ d	0.09	1.33	ns
A. curassavica	20	aphid	$\mu g = 647.5 - 28.3$ d	0.009	0.12	ns
A. curassavica	20	opposite	$\mu g = 760.9 - 81.5$ d	0.07	0.98	ns
A. curassavica	55	aphid	$\mu g = 1676.3 - 114.8$ d	0.11	1.65	ns
A. curassavica	55	opposite	$\mu g = 1197.1 - 65.9$ d	0.04	0.48	ns
A. curassavica	148	aphid	$\mu g = 2213.0 + 131.6$ d	0.02	0.29	ns
A. curassavica	148	opposite	$\mu g = 2228.3 - 140.2$ d	0.07	0.90	ns

little cardenolide in *A. incarnata* that there were insufficient data for regression analyses (Figures 2 and 3).

Regression analysis of leaf cardenolide content in *A. curassavica* against two measures of aphid biomass (natural logarithms of numbers and weight) all showed significant influences of increases in aphid biomass on leaf cardenolide (Figure 4). Cardenolide increased significantly with initial aphid density in both clip cage leaves and opposite leaves, and with final aphid density and biomass on clip cage leaves (Figure 4). There was also a significant regression relationship between cardenolide contents of opposite and clip cage leaves (Figure 5) with a slope of 0.58. This indicates that opposite leaves induced cardenolide production in concert with infested clip cage leaves but at a lower rate (slope <1). Despite the induction of both leaves, leaves with aphids in a clip cage had higher cardenolide contents (mean $=$ 1310 μg/g) than the opposite leaves (mean $=$ 1009 μg/g) ($t_{62} = 3.27,\ P = 0.002$).

DISCUSSION

We found that although aphid populations increased with time from each starting density (Table 2, Figure 1), there were no significant differences between aphid numbers or biomass on either host plant (Table 2). Thus, although the two host plants differed markedly in their constitutive cardenolide content, this difference had no impact on aphid numbers or biomass. Thus cardenolides in *A. curassavica* and *A. incarnata* had no measurable effect on aphid population dynamics. Interference competition for space within the clip cages may have limited the higher density treatments to a lower and more variable increase in aphid density with

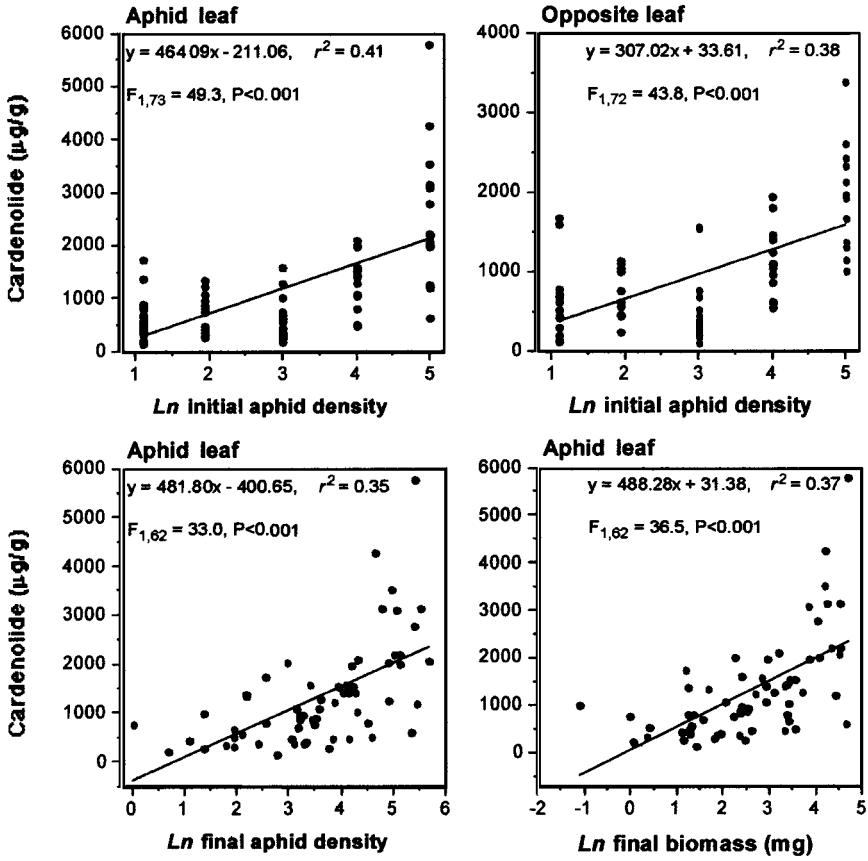

FIG. 4. Change in mean cardenolide concentrations in either aphid leaves or opposite leaves of *A. curassavica* with initial aphid density, final aphid density, or biomass.

time. Because *A. nerii* sequesters induced toxins for defense against natural enemies (Rothschild et al., 1970; Pasteels, 1978; Malcolm, 1981, 1986, 1989, 1990, 1992), intraspecific competition for these cardenolides may limit aphid population growth to a greater extent than competition for primary plant metabolites in phloem. The negative impact of such competition may be partially ameliorated from an evolutionary perspective by kin selection among apomictic relatives (Malcolm, 1986).

Similarly, we also found no significant effects of time on cardenolide expression in either *A. curassavica* or *A. incarnata,* measured in either leaves with aphids or adjacent, opposite leaves (Tables 3 and 4, Figures 2 and 3). We were surprised by this result because previous work with milkweeds (Malcolm and Zalucki, 1996)

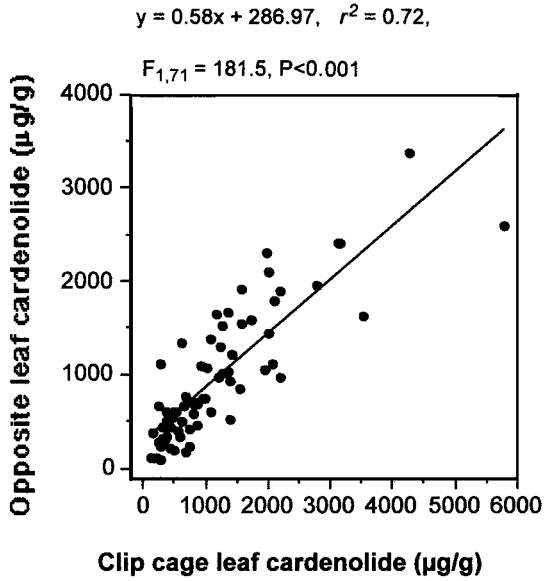

FIG. 5. Linear regression of cardenolide concentrations in opposite leaves against those in aphid leaves.

has shown that leaf damage to *A. syriaca* does induce cardenolide expression after 24 hr and that this expression decays back to approximately constitutive levels after 148 hr.

In contrast, we did find a significant effect of aphid density on cardenolide expression in *A. curassavica*, but not in *A. incarnata*. *A. incarnata* showed no measurable cardenolide response to aphids, but cardenolides in *A. curassavica* did respond to aphid density (Figure 4). The effect of aphid density on cardenolide expression by *A. curassavica* leaves surprised us because our *a priori* expectation was that the host with the lowest constitutive defense, *A. incarnata*, would induce cardenolide production more strongly in response to herbivory than the well-defended *A. curassavica* with high constitutive cardenolide. The rationale for this expectation was that low defense expression when herbivores are absent minimizes constitutive costs of defense, but that this low cost needs to be balanced by rapidly inducible defenses when a plant is attacked by herbivores. However, we found almost no detectable cardenolide in either control or aphid-infested *A. incarnata*.

Cardenolide contents increased significantly with initial aphid density in both clip cage leaves and opposite leaves, and with final aphid density and biomass on clip cage leaves from *A. curassavica* (Figure 4). Our data show that *A. curassavica* does have high constitutive levels of cardenolide in the absence of herbivores, but that cardenolide content actually *reduces* at low aphid densities, and then as aphid

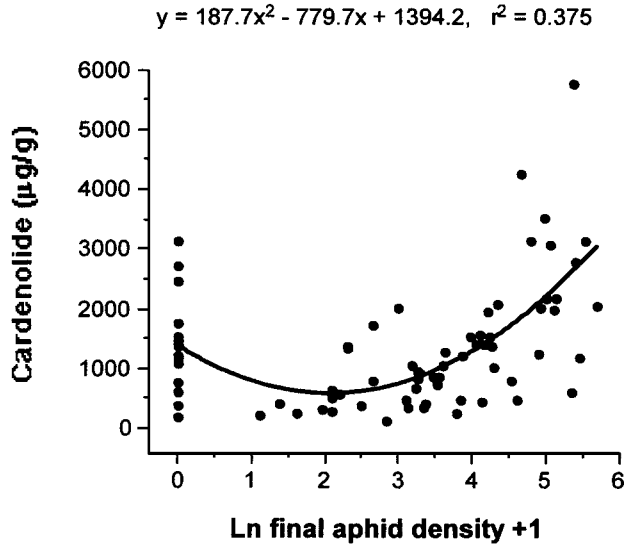

FIG. 6. Polynomial regression of leaf cardenolide content against aphid-free controls and final aphid densities.

density increases cardenolide content is induced, until at high aphid densities they are above the constitutive controls (Figure 6). Our data suggest a significantly curvilinear relationship that reflects both *reduction* and *induction* of cardenolides in response to aphid density as well as constitutive cardenolide in the absence of aphids.

Such a response, where dynamic defenses both *reduce* and *induce* in response to variable herbivore load, is important to plants that are attacked by specialist herbivores that can co-opt plant defenses by sequestration for use in their own defenses against natural enemies in the third trophic level. Reduction of plant defense can make sequestering herbivores more susceptible to natural enemies, so that more natural enemies can become "included" and increase herbivore mortality as a function of defense expression. Evidence for this scenario is described by Malcolm (1992) for this same system of *A. nerii*, *A. curassavica*, and a suite of different species of predators and parasitoids recorded as natural enemies of *A. nerii* around the world.

The response of aphid-infested leaves was reinforced by the response of uninfested leaves adjacent to the site of feeding (Figure 5). However, this response was lower than that of the infested leaves because leaves with aphids in a clip cage had significantly higher cardenolide contents (mean $= 1310$ μg/g) than opposite leaves (mean $= 1009$ μg/g). This suggests that defense reduction and induction occur systemically throughout a ramet, but that the dynamic response is strongest closest to the site of herbivory.

Hunter and Price (1992) suggested the possibility that particular herbivores may adversely affect both the first and third trophic levels. If herbivore-sequestered phytochemicals can reduce natural enemy efficacy, herbivore population density might increase and exert a direct negative impact on host-plant fitness, especially in cases where herbivores are not adversely affected by plant chemistry. Malcolm (1995) and Malcolm and Zalucki (1996) suggested the existence of a *lethal plant defense paradox* operating such that chemical defense mechanisms impose a negative impact on herbivores that may not necessarily impact host plants in a positive manner. If cardenolide expression in milkweeds is a function of *A. nerii* density, cardenolide induction could be both metabolically expensive and ineffective against herbivory. *A. nerii* might sequester the induced toxin for defense against its own natural enemies (Rothschild et al., 1970; Pasteels, 1978; Malcolm, 1981, 1986, 1989, 1990, 1992) or excrete what it is unable to assimilate in honeydew.

Karban and Baldwin (1997) suggested that phytochemical induction might rely, in part, on host-plant exposure to herbivore salivary β-glucosidase. Although *A. nerii* does not reduce *A. curassavica* foliar biomass, its piercing/sucking foraging strategy does expose plant tissue to salivary β-glucosidase and causes host damage at the cellular level (Dixon, 1998). Botha et al. (1977) photographed *A. nerii* stylets penetrating phloem sieve tubes and companion cells of the internal phloem in four milkweed species. A preference for the internal phloem of the bicollateral vascular bundles is evidence for the aphid's specialized feeding habit and the sink it represents for milkweed resources. Such specialized foraging activity is apparently sufficient to elicit a density-dependent inductive response in the cardenolides of *A. curassavica* but not in *A. incarnata*. This does not mean that *A. incarnata* is not defended by allelochemicals, and it is possible that *A. incarnata* has shifted to reliance on different, but biochemically related chemical defenses, such as the steroidal pregnane glycosides suggested by Roeske et al. (1976).

The impact of haustellate aphid herbivory on plant defense expression is poorly understood. Although aphid herbivory typically does not reduce host-plant foliar biomass, it is a resource sink and may reduce simple sugar and amino acid concentrations in phloem sap. To fuel extraordinarily high population growth rates, phloem-feeding aphids must process particularly large quantities of food due to low nitrogen concentrations in plants (Dixon, 1970, 1998; McNeill and Southwood, 1978). Therefore, the foraging activities of aphids in dense colonies should lower host-plant fitness. This in turn suggests the likely operation of selection for defenses to protect plant fitness. Such a response was described by Muller (1959), who found that the roots of grape, *Vitus vinifera*, induce the production of "corky" necrotic zones in response to feeding by the vine louse, *Phylloxera vasatrix*. These zones function to isolate the herbivore from remaining healthy root tissue. More recently, Gianoli and Niemeyer (1996, 1997) found that wheat, *Triticum aestivum*, induces hydroxamic acid in response to feeding by a minimum of 25 bird-cherry

aphids, *Rhopalosiphum padi*, following 48 hr of infestation. Thus, it is possible that milkweeds may also engage in damage-induced cardenolide expression in response to piercing/sucking herbivory by *A. nerii*.

Despite the fact that *A. curassavica* induces cardenolides as a function of *A. nerii* density, the insect population data suggest that milkweed secondary chemistry exerts little adverse effect on herbivore colony biomass or population density, over the short-time period of our measurements. *A. curassavica* and *A. incarnata*-reared *A. nerii* colony biomass and population densities were virtually identical. This again suggests that *A. nerii* is an adapted specialist that largely avoids the deleterious effects of milkweed-induced resistance, regardless of cardenolide concentration. Because cardenolide sequestration reduces predator efficacy (Malcolm, 1981, 1986, 1989, 1990, 1992), cardenolide induction in some milkweed species may enhance the effectiveness of *A. nerii*-sequestered chemical defense against natural enemies in the third trophic level.

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ANTIFEEDANT COMPOUNDS FROM THREE SPECIES OF APIACEAE ACTIVE AGAINST THE FIELD SLUG, *Deroceras reticulatum* (MULLER)

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Abstract—Extracts of volatiles from foliage of three plants in the Apiaceae, *Conium maculatum* L. (hemlock), *Coriandrum sativum* L. (coriander), and *Petroselinum crispum* Mill. (Nym.) (parsley), previously shown to exhibit antifeedant activity in assays with the field slug, *Deroceras reticulatum* (Muller) (Limacidae: Pulmonata), were studied further to identify the active components. Coupled gas chromatography–mass spectrometry (GC–MS) and neurophysiological assays using tentacle nerve preparations resulted in the identification of 11 active compounds from the three extracts. Wheat flour feeding bioassays were used to determine which of these compounds had the highest antifeedant activity. One of the most active compounds was the alkaloid γ-coniceine, from *C. maculatum*. The role of potentially toxic alkaloids as semiochemicals and the potential for using such compounds as crop protection agents to prevent slug feeding damage is discussed.

Key Words—*Deroceras reticulatum*, *Conium maculatum*, *Coriandrum sativum*, *Petroselinum crispum*, Apiaceae, neurophysiology, bioassay, γ-coniceine.

INTRODUCTION

Slugs, particularly the field slug, *Deroceras reticulatum* (Muller) (Limacidae: Pulmonata), are prolific pests in both agriculture and horticulture. The most

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important economic damage is caused by direct feeding in the form of cereal grain hollowing and defoliation. Molluscicidal baits currently are used to reduce slug populations and minimize feeding damage. However, because of the unreliable nature of these baits, and the risk posed to nontarget organisms, alternatives for slug control are desirable. This has encouraged research into various means of reducing crop damage by slugs without recourse to the use of toxic modes of action. One promising approach lies in the use of semiochemicals (behavior-modifying chemicals), particularly naturally occurring, plant-derived repellents or antifeedants (Dawson et al., 1996; Dodds, 1996; Dodds et al., 1996; Powell and Bowen, 1996). The potential of plant-derived antifeedants was originally demonstrated in screening studies of plant extracts versus *D. reticulatum* (Airey et al., 1989; Garraway, 1992; Clark et al., 1997), from which the volatile monoterpenoid (+)-fenchone was identified as a compound that significantly reduced feeding.

Seminal studies on *D. reticulatum* chemoreception established that both posterior and anterior tentacles are associated with feeding and orientation behaviors. The posterior tentacles are involved with olfaction of volatile materials whereas the anterior tentacles are used for contact chemoreception (Stephenson, 1979; Chase, 1981, 1982; Chase and Croll, 1981). Using this knowledge, a neurophysiological assay was developed to record the olfactory responses of *D. reticulatum* from the posterior tentacle (Garraway, 1992). Recordings from the tentacular olfactory nerve of the isolated posterior tentacle were made by suction electrodes, with an airstream containing volatile extracts or compounds directed over the exposed sensory pad. Using this assay, it was shown that (+)-fenchone strongly stimulated the tentacular nerve of *D. reticulatum*. The nerve preparation also discriminated in its response between (+)- and (−)-isomers of fenchone, as well as various other natural products, and further, repetitive applications of the same compound produced a consistent effect (Garraway et al., 1991). These observations led to the use of (+)-fenchone as a standard stimulus to monitor preparation activity, so that the response to other compounds could be normalized (Garraway et al., 1992).

Because (+)-fenchone is a common component of plants in the family Apiaceae (Umbelliferae), a wider survey was conducted within this plant family to search for other compounds that reduce slug feeding (Dodds, 1997; Dodds et al., 1999). Foliar extracts of various species were found to reduce slug feeding when applied to a standard food source. Within the range of 17 species of Apiaceae screened, 3 species were consistently more effective, suppressing slug feeding by over 60%. Therefore, it was considered that these three species, *Conium maculatum* L. (hemlock), *Coriandrum sativum* L. (coriander), and *Petroselinum crispum* (Mill.) Nym. (parsley), all contained phytochemicals that interfered with normal feeding behavior. The aim of the current study was to assess the neurophysiological activity, using the isolated tentacle assay (Garraway et al., 1992), of *C. maculatum*, *C. sativum*, and *P. crispum* extracts, and to isolate and identify the

active compounds, with a view to their use as crop protection agents. The biological activity of the components was recorded using an established bioassay which formally measures antifeedant activity (Clark et al., 1997). Because (+)-fenchone had been identified previously as having antifeedant activity against *D. reticulatum,* compounds with similar physical properties were mainly investigated here.

METHODS AND MATERIALS

Test Animals. Adult field slugs, *Deroceras reticulatum* (weight range 400–600 mg), were collected from the grounds of Rothamsted, from beneath laminated cork/polystyrene roof insulation boards (approximately 1 m^2), on an irrigated plot of mixed herbage. They were maintained in plastic bowls lined with moist cotton wool covered with absorbent, unbleached paper in a controlled environment (L:D 12:12, 15°C light, 5°C dark), and fed on Chinese cabbage (*Brassica chinensis* L. *cv.* tip-top) for at least 24 hr, then starved for a further 24 hr before being used in the neurophysiology studies and feeding bioassays.

Plant Extraction. The three species of Apiaceae tested, *Conium maculatum* L. (hemlock), *Coriandrum sativum* L. (coriander), and *Petroselenum crispum* (parsley), were all grown from seed (Chiltern Seeds, UK), and extracted just prior to flowering. Plant material was extracted using microwave-assisted distillation, based on a previously reported method (Craveiro et al., 1989). Fresh weight material (30–40 g leaf material only, because stem extraction leads to the release of artefacts via pyrolysis) was heated in a 500-ml florentine flask for approximately 1 min in an 800-W microwave oven until the plant cells ruptured. The released volatile chemicals were carried in a stream of high purity nitrogen at a flow rate of 60 ml/min through polytetrafluoroethylene (PTFE) tubing (3 mm i.d.), and trapped in a Drechsel bottle containing cooled (ice-bath), freshly distilled hexane (50 ml). The extracts were dried with anhydrous $MgSO_4$, then filtered and concentrated *in vacuo* to the equivalent of 25 g fresh weight plant material per milliliter hexane prior to quantification and use in bioassays. Extracts were stored in microvials at −20°C until further use.

Gas Chromatography (GC). Plant volatile extracts were separated on an HP5890A gas chromatograph equipped with a cool on-column injector, a flame-ionization detector (FID), and an HP-1 fused silica capillary column (50 m × 0.32 mm i.d., 2.65 μm film thickness). The oven temperature was held at 40°C for 1 min, then programmed at 5°C/min to 150°C, then 10°C/min to 250°C. The carrier gas was hydrogen. Quantities of biologically active components were calculated from the individual peak areas and response factors for the individual compounds. Response factors were calculated from injections of known amounts of each compound (100 ng) containing an internal standard (100 ng *n*-tridecane)

apart from ocimene, which was calculated by comparison of peak areas with other monoterpenes.

Coupled GC–Mass Spectrometry (GC–MS). A capillary GC column (50 m × 0.32 mm i.d., HP-1) fitted to a cool on-column injector was directly coupled to a mass spectrometer (VG Autospec, Fisons Instruments). Ionization was by electron impact (70 eV, 200°C). The oven temperature was held at 30°C for 5 min, then programmed at 5°C/min to 250°C. The carrier gas was helium. Tentative identifications were confirmed by peak enhancement with authentic samples (Pickett, 1990).

Chemicals. Chemicals identified as components of *C. maculatum*, *P. crispum*, and *C. sativum*, and which were subsequently screened in neurophysiological and behavioral assays, were obtained from commercial sources (Aldrich Chemical Company; Fluka Chemie AG; Sigma-Aldrich Company Ltd.; >97% purity by GC). Where relevant, chiral forms of chemicals were used in assays, i.e., (+)-*trans*-limonene-1,2-epoxide and (+)-3-carene. γ-Coniceine (>93% pure) was synthesized (114 mg, 63% over two steps) from (±)-coniine (Grundon and Reynolds, 1964). The identity was confirmed by comparison of ^{1}H NMR and MS data with literature values (Fukuda et al., 1991). For neurophysiological and behavioral assays, solutions of chemicals in freshly distilled hexane were prepared at the concentrations at which they were found in the plant extracts.

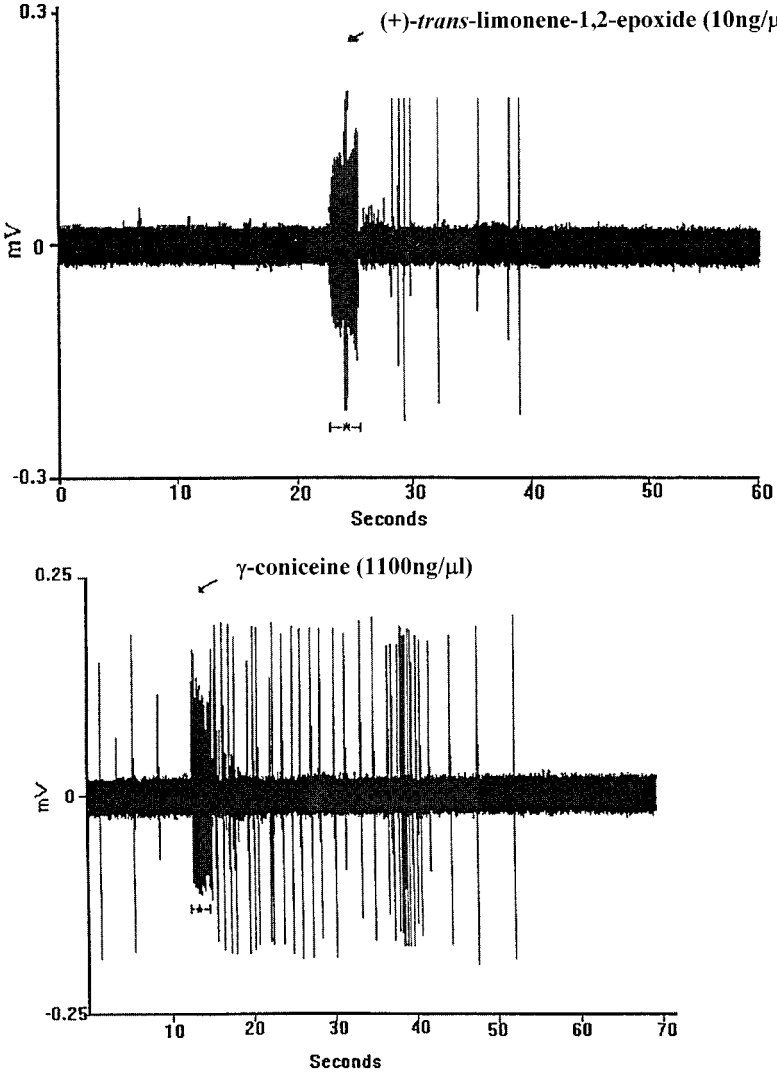
Electrophysiology. A posterior tentacle was dissected out of a CO_2-anesthetized slug, and pinned in a Sylgard dish containing a specially formulated Ringer solution (Garraway, 1992) (NaCl 70 mM; KCl 2.5 mM; $CaCl_2 \cdot 6H_2O$ 3.5 mM, $MgCl_2$ 1.5 mM, $NaHCO_3$ 10 mM; KH_2PO_4 0.8 mM, glucose 50 mM; pH 7.53). The tentacle was dissected to expose further both the main olfactory nerve and the sensory pad, and positioned so that the nerve was immersed in the Ringer solution while the pad remained exposed to air. Recordings were made from the olfactory nerve, using suction electrodes, while a purified, humidified airstream (60 ml/min) was directed over the sensory pad through a 1 mm diameter metal tube.

Aliquots (2 μl) of each test compound in hexane were delivered into the airstream via an injection port and over the sensory pad of the same nerve preparation in a random order, with a 10-min resting period between dosing. Hexane controls (2 μl) were also used. A (+)-fenchone standard solution (10 ng/μl, 2 μl) was tested at random intervals to eliminate the possibility of desensitization of the preparation with time. Because of the difficulty in making active preparations, only three replicates were tested. Electrical activity elicited in the preparation by test stimuli was amplified by a DAM50 differential amplifier (World Precision Instruments). Amplified electrical responses were then simultaneously displayed on a digital storage oscilloscope ("Wavetek" 20 MHz) and recorded on magnetic tape for subsequent computer analysis using appropriate software (Spike 2, Cambridge Electronic Design (CED)).

Some compounds induced a characteristic electrophysiological response by the nerve preparation (Figure 1). The rate of firing of the nerve was increased for a finite period (the "envelope" of the response) before it returned to the resting point. During the firing period, the firing frequency was not constant, being greater at the onset of the response. Thus, three parameters were chosen to measure the intensity of the response: (1) duration of whole response (in sec) during which activity remained above spontaneous level; (2) total number of events, i.e., action potentials (APs) during this period of increased frequency; and (3) increase in frequency for the first 10 sec after stimulation. When spontaneous activity levels were high before the test compound was applied, it was difficult to measure the changes in the frequency of the spikes produced in response to the compound. To overcome this, spike frequency was measured for approximately 50 sec prior to stimulation, ignored during the stimulus artefact period, and then measured over a fixed period of ten sec after the extract was applied. The changes were analyzed using Spike 2 software (CED).

Statistical analysis of the electrophysiology data was carried out by calculating mean values for each neurophysiologically active compound, based on three replicates, for each of the variables (duration of nerve response, total number of APs, and increase in frequency of firing during the first 10 sec after stimulus application), and performing Principal Components Analysis (PCA) using these variables, along with the mean antifeedant response, as reported previously (Dodds et al., 1999).

Behavioral Bioassays. A wheat flour pellet feeding bioassay was used as described previously (Clark et al., 1997). Solutions of the compounds identified from *C. maculatum*, *C. sativum*, and *P. crispum* were prepared in freshly distilled hexane, and were dosed individually onto wheat flour pellets (30 μl per pellet). Control pellets were dosed with distilled hexane (30 μl). The preweighed wheat flour pellets with either single components or the control were placed on to filter papers (Whatman No. 1) in Petri dishes (9 cm diam, one pellet per dish). The dishes were placed in to a controlled environment as described above (see *Test Animals* section), and the filter papers were moistened with distilled water. Single *D. reticulatum* slugs, previously starved for 24 hr, were placed into each of the dishes, and stainless steel lids (0.5 mm mesh) were placed on top to contain the slugs, while also preventing build up of vapor from volatile test materials. After 24 hr, the slugs were removed and each pellet was dried to constant weight at 50°C. The dry weight of each pellet consumed was calculated by difference. Each compound was tested 30 times. Statistical analysis was carried out as described previously by ANOVA and the difference in feeding (C), i.e., the difference in mean weights consumed by the test ($\bar{T}$) and control ($\bar{U}$) slugs was expressed as a percentage with a standard error (Clark et al., 1997). Because C is the ratio of two measured variables ($C = 100(\bar{T} - \bar{U}/\bar{U})$, and $\bar{T}$ and $\bar{U}$ are independent means, its



* represents an artefact brought about by the syringe delivering the stimulus being placed within the recording environment.

FIG. 1. Typical responses from olfactory nerve preparations of *Deroceras reticulatum* in response to γ-coniceine and (+)-*trans*-limonene-1,2-epoxide identified in *Conium maculatum* (hemlock). Traces are examples of high and low activity, with γ-coniceine inducing a more intense response than the (+)-*trans*-limonene-1,2-epoxide.

standard error was calculated as

$$\mathrm{SE}(C) = 100\sqrt{[\{\mathrm{Var}(\bar{T}) + \mathrm{Var}(\bar{U})\}/\bar{U}^2 + (\bar{T} - \bar{U})^2\mathrm{Var}(\bar{U})/\bar{U}^4 + 2(\bar{T} - \bar{U})\mathrm{Var}(\bar{U})\bar{U}^3]}$$

where $\mathrm{Var}(\bar{T}) = \mathrm{Var}(\bar{U}) = \sqrt{s^2/n}$, s^2 being the residual mean square from the analysis of the variance and n the replication (30).

RESULTS

Coupled GC–MS analysis, followed by comparison with reported spectra (NIST, 1990; Fukuda et al., 1991) and peak enhancement on GC led to the identification of several major components within extracts of *C. maculatum*, *C. sativum*, and *P. crispum* (Table 1). Peaks eluting very early or late in the GC traces were not identified in order to concentrate on compounds with physical properties in the range of the known antifeedant (+)-fenchone. Quantities of the identified major components were calculated using *n*-tridecane as an internal standard (Table 1).

TABLE 1. MAJOR COMPONENTS IN *Conium maculatum* L. (HEMLOCK), *Coriandrum sativum* L. (CORIANDER), AND *Petroselinum crispum* MILL. (NYM.) (PARSLEY) EXTRACTS PREPARED BY MICROWAVE-ASSISTED DISTILLATION

Extract	Compound	Concentration (μg/ml)[a]
C. maculatum	β-Pinene	15
	Myrcene	150
	γ-Coniceine	1100
	Ocimene[b]	508
	trans-Limonene-1,2-epoxide[c]	10
	β-Caryophyllene	9
	p-Cymene	20
C. sativum	Decanal	0.26
	(*E*)-2-Decenal	1100
	(*E*)-2-Undecenal	0.12
	(*E*)-2-Dodecenal	0.11
	2-Tetradecenal[d]	—
P. crispum	Myrcene	0.10
	3-Carene[c]	0.22
	1,3,8-*p*-Menthatriene[d]	—
	Myristicin	0.18

[a] Peaks quantified using *n*-tridecane as an internal standard.
[b] (*E*)- and (*Z*)-isomers combined.
[c] Stereochemistry undefined.
[d] Tentative identification only.

For *C. maculatum*, seven major volatile components were identified, one of which gave the following major ions, m/z (intensity): 125 (15%), 110 (32%), 97 (100%), 96 (30%), 82 (16%), 70 (27%), 55 (17%), 54 (16%), and 41 (32%), consistent with the piperideine alkaloid γ-coniceine. The absolute stereochemistry of *trans*-limonene-1,2-epoxide was not determined. For *C. sativum*, four aldehydes were identified, plus another aldehyde, 2-tetradecenal, for which identification remained tentative because an authentic sample was not available. For *P. crispum*, three major components were identified, plus tentatively, 1,3,8-*p*-menthatriene, for which an authentic sample was not available. The stereochemistry of 3-carene was not determined.

To establish which of the major components in the extracts elicited neurophysiological activity in *D. reticulatum*, the individual identified components from *C. maculatum*, *C. sativum*, and *P. crispum* were tested on three tentacle nerve preparations at the concentration at which they occurred in extracts. All compounds identified in *C. maculatum* were more stimulatory than the control, apart from ocimene, to which no significant response was observed (Figure 2). Responses for all components varied considerably in intensity among the three screenings of a single component, but no differences were observed in the rank order of activity, with γ-coniceine inducing a greater level of activity, with more frequent APs over a short period of time, and (+)-*trans*-limonene-1,2-epoxide inducing fewer APs. For *C. sativum*, the four aldehydes identified were more stimulatory than the control, inducing similar levels of electrical activity in the nerve preparation. However, the responses, were generally lower than those induced by active components in *C. maculatum*. For *P. crispum*, myrcene had already been shown to be stimulatory because it was also present in *C. maculatum*. (+)-3-Carene did not induce any response, whereas myristicin was highly stimulatory.

Individual compounds which induced nervous activity were screened in a wheat flour pellet bioassay to measure their antifeedant activity with intact *D. reticulatum* slugs. For *C. maculatum*, the compounds were tested at the concentrations at which they occurred within the extracts. All compounds showed significant antifeedant activity compared to the control, with γ-coniceine showing the highest activity, reducing feeding by $70 \pm 5.4\%$ (Figure 3). All of the active compounds, including those from *C. sativum* and *P. crispum*, were then tested at an identical dose (1000 μg/ml), to determine which of the compounds were most effective by direct comparison (Figure 4). No significant differences were observed in the activity of the compounds, except for (+)-*trans*-limonene-1,2-epoxide, which was less active than the other compounds. For *C. sativum*, all four aldehydes reduced feeding to a similar extent. For *P. crispum*, myristicin reduced feeding by $69 \pm 4.3\%$.

Principal Component Analysis (PCA) of the electrophysiology and antifeedant data for the neurophysiologically active compounds from *C. maculatum* showed a strong correlation between the mean values. Of the active compounds

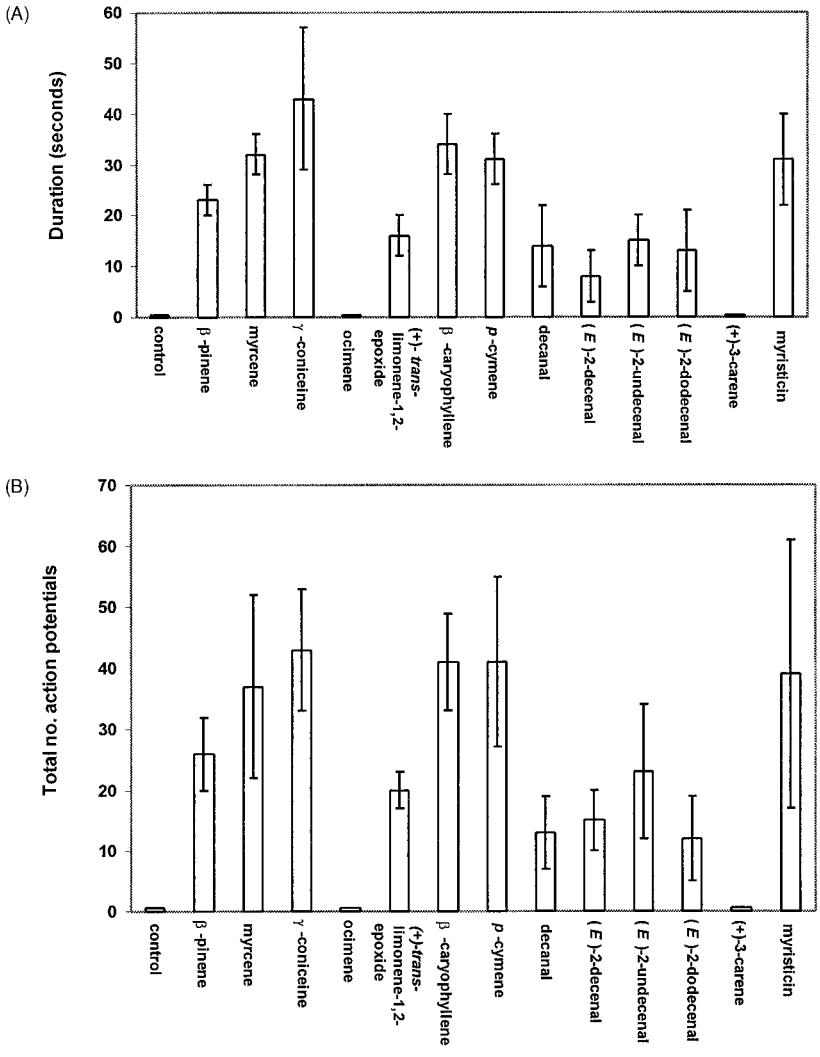


FIG. 2. Electrophysiological activity of olfactory nerve preparations of *Deroceras reticulatum* in response to volatile compounds from extracts of *Conium maculatum* (hemlock), *Coriandrum sativum* (coriander), and *Petroselinum crispum* (parsley). Compounds were tested at the concentration found in the extract. Error bars are expressed as standard error of the mean ($N = 3$). (A) Duration of the whole response; (B) total number of action potentials (APs) recorded following exposure; (C) increase in AP frequency during the first 10 sec after dosing. All responses, except those to ocimene and (+)-3-carene, were significantly higher than controls.

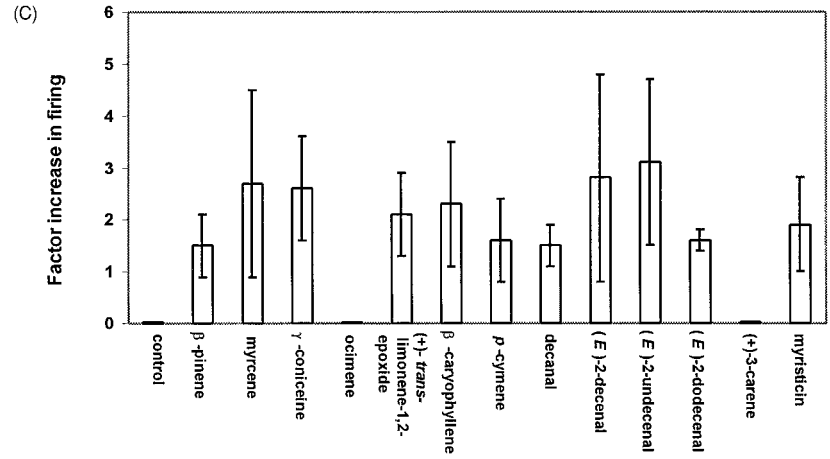


FIG. 2. (CONTINUED).

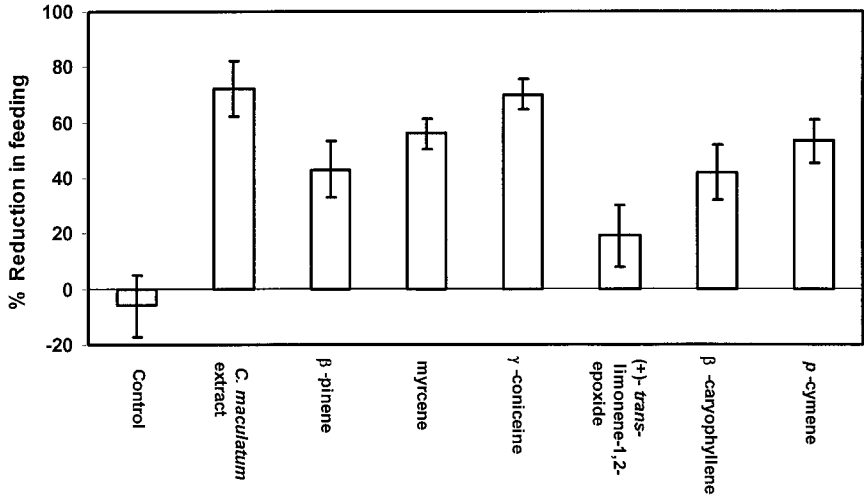


FIG. 3. Effect of volatile compounds of *Conium maculatum* (hemlock) extract on percentage reduction in feeding of *Deroceras reticulatum*, compared to control stimulus (hexane). Compounds were tested at concentrations found in the extract. Error bars are expressed as standard error of the mean ($N = 30$).

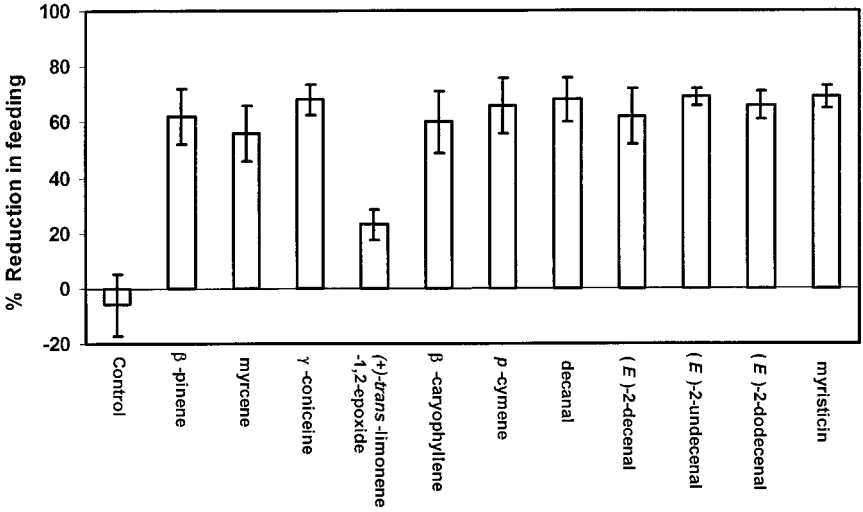


FIG. 4. Effect of volatile compounds of *Conium maculatum* (hemlock), *Coriandrum sativum* (coriander), and *Petroselinum crispum* (parsley) extracts on feeding behavior of *Deroceras reticulatum* at a standard concentration (1000 μg/ml), compared to a control stimulus (hexane). Error bars are expressed as standard error of the mean ($N = 30$).

tested, (+)-*trans*-limonene-1,2-epoxide was least active and γ-coniceine was most active. Other compounds from *C. maculatum* were shown to have moderate neurophysiological and antifeedant activity.

DISCUSSION

Sixteen components from extracts of *C. maculatum*, *C. sativum*, and *P. crispum*, shown previously to have antifeedant activity against *D. reticulatum*, were isolated and identified by coupled GC-MS. Of these, 11 elicited activity in the tentacle nerve preparation when applied at the concentration at which they occurred in plant extracts. A sample of (*E*)- and (*Z*)-ocimene did not stimulate the tentacle preparation, even at a high concentration (508 μg/ml), nor did (+)-3–carene (0.22 μg/ml). Responses to other compounds were recorded at concentrations as low as 9 μg/ml, as exemplified by β-caryophyllene.

Little work has been carried out on the threshold levels at which components are perceived by chemoreceptors of molluscs, particularly slugs. Of the invertebrates, insects have been shown to be able to respond at the single molecule level to pheromones (Kaissling, 1986). However, the smallest amount of (+)-fenchone found to be detected by the tentacle preparation of *D. reticulatum* was 10 pg (Garraway, 1992).

When individual compounds were tested on nerve preparations from different slugs, the intensity of the response varied considerably. However, the overall rank order of activity, defined as the duration of response and the number of APs, remained similar. In all cases, the total duration and intensity of the response was reduced compared to that triggered by the entire extracts (Dodds et al., 1999), suggesting that the neurophysiological activity of the extracts resulted from perception of several different components. The sensitivity of the preparation was relatively stable because responses induced by application of the (+)-fenchone standard (20 ng) remained constant throughout the testing period. This was consistent with a previous study (Garraway, 1992) and confirmed the reliability of the assay. When the compounds identified from the *C. maculatum* extract were tested at the levels found in the extract, all compounds consistently induced activity (duration of response, Figure 2A) in the tentacle nerve preparation. However, because compounds were not tested at standard concentrations, it was not possible to compare activity directly and determine which ones were most active. Nevertheless, it was clear that the slug tentacle preparations responded to components at the levels found in the plants.

In general, the individual compounds were less stimulatory than whole plant extracts, as illustrated by the *C. maculatum* extract and identified compounds, indicating that the biological activity of an extract is likely to be the result of the combination of several compounds (Dodds et al., 1999). In the current study, the sum of the total number of APs within an individual response, and their frequencies within the first 10 sec of those responses, approximated the response to the whole extract. However, the total duration of the responses to extracts were nearly twice as long as the sum of those to the individual compounds, suggesting that there may be synergy among compounds within an extract. Application of individual components of the extracts also induced APs of several different amplitudes, suggesting that each compound was acting on more than one axon type. PCA of the electrophysiological data showed a strong correlation between electrophysiological and antifeedant activity for compounds (data not shown), in a manner similar to that reported previously for plant extracts (Dodds et al., 1999), thus showing that the neurophysiological assay is a useful screening technique for determining the possible biological activity of plant-derived antifeedants for slugs.

When the active components of *C. maculatum* were screened in the feeding bioassay at the concentrations at which they occurred within extracts, the major compound, γ-coniceine, resulted in the highest antifeedant activity. The compounds were also tested, along with those identified from *C. sativum* and *P. crispum*, at a standard concentration (1000 μg/ml) to give an indication of how they would perform comparatively for any subsequent field studies. Under these conditions, compounds showed little difference in activity, apart from (+)-*trans*-limonene-1,2-epoxide, which had lower activity. γ-Coniceine was still one of the most potent antifeedants.

Previous work on the identification and activity of naturally occurring antifeedants has targeted mainly insects (Bettolo, 1983; Narwott et al., 1986; Bernays and Chapman, 1994). More recently, molluscan antifeedants have begun to receive attention (Watkins et al., 1996; Dodds, 1997). The repellent and antifeedant activity to slugs of phenolics, aromatics, and monoterpenes, when applied as treatments on wheat seeds, has been studied (Powell and Bowen, 1996). The chemicals screened were all naturally occurring compounds previously shown to modify insect behavior. Interestingly, (+)-limonene, which had antifeedant activity, was not repellent, thereby demonstrating the difference between repellent and antifeedant activity. Whereas a slug may pass over a surface coated with antifeedant material without being stressed, feeding will still be significantly reduced.

In summary, this study showed that naturally occurring plant compounds can act as antifeedants for *D. reticulatum*. Field studies are required to assess the potential for using such compounds in controlling slug populations in arable crop situations. Although all active chemicals significantly reduced slug feeding compared to controls, those compounds conveying the characteristic "smell" of the plants had the greatest antifeedant effect, rather than compounds associated with the ubiquitous green leaf character of plants. One of the most active compounds, γ-coniceine, is an alkaloid normally associated with relatively high toxicity, and is likely to be molluscicidal at higher concentrations. However, these phenomena would not be observed under natural conditions because of the potent antifeedant effects of γ-coniceine.

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DEFENSIVE CHEMICALS OF TWO SPECIES OF *Trachypachus* MOTSCHULSKI

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Abstract—Analyses of pygidial gland contents of two species of a previously uninvestigated family of beetles (Trachypachidae) by Gas Chromatography-Mass Spectrometry (GC-MS) revealed that their chemistry is similar to that reported from many members of the family Carabidae. Nevertheless, the composition of defensive gland fluids of the two species *Trachypachus slevini* and *T. gibbsii* differs sufficiently to distinguish between the two species solely on the basis of their defensive chemistry. The major components of *T. slevini* glandular fluid are methacrylic, tiglic, and octanoic (= caprylic) acids, together with the hydrocarbon (*Z*)-9-pentacosene. In contrast, the glandular contents of *T. gibbsii* contain a rather unique mixture of polar and nonpolar compounds, the principal constituents of which are methacrylic and ethacrylic acids (= 2-ethylacrylic acid), together with 2-phenylethanol, 2-phenylethyl methacrylate, 2-phenylethyl ethacrylate, and (*Z*)-9-pentacosene.

Key Words—Defensive secretion, methacrylic acid, tiglic acid, octanoic acid, Trachypachidae, *Trachypachus slevini*, *Trachypachus gibbsii*.

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INTRODUCTION

The extraordinary repertory of defensive chemicals used by beetles has undoubtedly contributed to their widespread success in terrestrial ecosystems (Meinwald and Eisner, 1995). Adephaga, the second largest suborder of beetles (Coleoptera), comprising approximately 40,000 species, is the best investigated group both at the species level and across families. Chemical studies on more than 500 species of Adephaga show that their secretions are rather complex mixtures of polar and nonpolar compounds (Blum, 1981; Dettner, 1987; Will et al., 2000). We recently studied two species of the previously uninvestigated adephagan beetle family Trachypachidae. We report here on the chemical composition of the pygidial gland defensive fluid of *Trachypachus slevini* Van Dyke and *Trachypachus gibbsii* LeConte, suggesting that both species have chemical defensive strategies similar to those that have been used by another adephagan family, Carabidae. This implies a possible close relationship between the two families.

Trachypachidae is one of the smallest families of Coleoptera with only two genera, *Trachypachus* Motschulski and *Systolosoma* Solier. The family's amphitropical distributional pattern is disjunct and relictual. The four species of *Trachypachus* are distributed in western Nearctic (three species) and eastern Palearctic (one species) regions. *Systolosoma* has two described species restricted to Chile and Argentina.

The monophyly of Trachypachidae and its membership in the suborder Adephaga are well supported (Beutel, 1995). Its relationship to other adephagan families, however, is contentious. Some analyses place them as a sister group of Carabidae (e.g., Kavanaugh, 1986; Shull et al., 2001). Other authors, on the basis of various character systems, place them as a sister group to all or part of the aquatic taxa in Hydradephaga (Bell, 1966; Arndt, 1993; Deuve, 1993; Beutel and Haas, 2000). Although a variety of morphological characters have been studied, the hypothesized direction of character change and significance have been challenged (Kavanaugh, 1986). To date, only a single gene sequence, using one species from each trachypachid genus, has been investigated for familial level relationships in Adephaga (18S ribosomal RNA, Shull et al., 2001).

Pygidial gland secretions are ecologically and phylogenetically significant (Moore and Brown, 1979; Dettner, 1985, 1987; Will, 2000; Will et al., 2000). With this in mind, we have identified the volatile constituents of pygidial glands in *T. slevini* and *T. gibbsii* and have interpreted them in terms of phylogeny and ecology. A number of compounds that we identified from these beetles are significant as secondary metabolites because of their scattered and rare occurrence in arthropods.

METHODS AND MATERIALS

Insects. Live beetles were collected in California, Oregon, and Washington states and transported to laboratory facilities in Berkeley, California, and Tucson,

Arizona. Beetles were kept cool and fed both chopped mealworm larvae (*Tenebrio*) and commercial dog food. Dissection and secretion sampling methods followed Will et al. (2000). Sealed glass ampoules containing excised gland reservoirs were shipped to laboratory facilities in Ithaca, New York or Hoboken, New Jersey for chemical analysis. Ampoules containing the gland reservoirs were kept frozen until chemical analysis could be performed.

Reference Compounds and General Procedures. Ethacrylic acid, isopropyl methacrylate, isopropyl ethacrylate, 2-phenylethyl methacrylate, and 2-phenylethyl ethacrylate were synthesized. Other reagents were available in our collection of chemicals, or purchased from Aldrich Chemical Company. Synthetic reactions were monitored by TLC. Electron-ionization (EI) mass spectra were measured by GC-MS using an HP 5890 II gas chromatograph (GC) linked to an HP 5989A mass spectrometer. Infrared spectra of synthetic compounds were measured on a Perkin–Elmer Paragon 1000 PC Fourier Transform Infrared (FT-IR) spectrometer.

Analytical Procedures. For gas chromatographic analysis of glandular extracts of *T. slevini*, the tubes were opened, and each sample was extracted with 20 μl of dichloromethane or hexane, and a 0.5 μl aliquot of each sample was analyzed by GC-MS using an HP 5890 GC linked to an HP 5970 mass selective detector [25 m $\times$ 0.22 mm fused-silica column coated with HP-1; the oven temperature was kept at 60°C for 4 min and raised 10°C/min to 260°C], or a Micromass Autospec mass spectrometer [Analyses were performed using a 25 m $\times$ 0.25 mm fused-silica column coated with 0.25 μm ZB-FFAP (nitroterephthalic acid modified polyethylene glycol; Phenomenex, Torrance, CA)]. The oven temperature was kept at 40°C for 3 min and raised 6°C/min to 265°C].

Gland reservoir contents of *T. gibbsii* were extracted with hexane (20 μl) and 1 μl of the extract was injected into the GC-MS [HP 5989A mass spectrometer linked to an HP 5890 II GC equipped with a ZB-FFAP-coated (0.25 μm) fused-silica capillary column (30 m $\times$ 0.25 mm)]. The oven temperature was held at 30°C for 2 min and increased at 6°C/min to 240°C.

GC-FTIR analyses were performed using an Infrared Detective Instrument (Bourne Scientific, Acton, MA) linked to a Shimadzu 17A GC. The GC was fitted with a 15 m $\times$ 0.25 mm fused-silica column coated with RTX5 (0.25 μm). Compounds eluting from the GC column were passed through a transfer line (300°C) and frozen on a rotating ZnSe window (speed: 2 mm/min) at -65°C, and transmission FT-IR spectra were recorded.

*Synthesis of Ethacrylic Acid (= 2-Ethylacrylic Acid) (**3**).* Ethyl 2-ethylacrylate (**2**): A solution of triethyl phosphonoacetate (**1**, 0.341 ml, 1.68 mmol; 98% purity) in tetrahydrofuran (0.5 ml) was added dropwise to a suspension of NaH (67.4 mg, 1.69 mmol) in mineral oil (60%) at 0°C. The reaction mixture was stirred at room temperature for 0.5 hr, cooled to 0°C, and EtI (0.136 ml, 1.69 mmol) was added dropwise and stirred at room temperature for 14 hr. A further aliquot of NaH (67.4 mg, 1.69 mmol, 60% in mineral oil) was added at 0°C, and the reaction mixture was stirred at room temperature (1 hr). Paraformaldehyde (58.6 mg,

1.85 mmol, 95% purity) was added at room temperature, and the mixture was stirred for 1 hr. After diluting with H_2O (1 ml), the mixture was extracted with pentane (3×0.5 ml). Combined pentane extract was washed with H_2O (2×0.5 ml) and evaporated to about 0.25 ml and used in the next step. Ethyl 2-ethylacrylate (**2**): EIMS m/z: 128[M]$^+$ (8), 113 (19), 100 (18), 95 (3), 85 (13), 83 (52), 82 (29), 69 (3), 55 (100), 45 (18), 43 (9), 39 (22).

*2-Ethylacrylic Acid (**3**)*: A mixture of **2**, NaOH (75 mg, 1.875 mmol), MeOH (0.5 ml), and H_2O (0.5 ml) was stirred on an oil bath at 100°C for 4.5 hr. Methanol was evaporated, and the mixture was acidified (Conc. H_2SO_4 0.1 ml + 0.5 ml water), and saturated with NaCl. The product was extracted into CH_2Cl_2/pentane (1:1; 3×0.5 ml) and dried over $MgSO_4$. The solvent was evaporated to give a colorless liquid **3** (102 mg, 59% total yield). IR (neat) ν_{max} 2975, 1698, 1629, 1446, 1194 cm^{-1}; EIMS m/z: 100 [M]$^+$ (47), 99 (4), 85 (29), 82 (25), 73 (7), 72 (9), 60 (5), 55 (100), 50 (11), 45 (23), 44 (24), 39 (48).

Synthesis of 2-Phenylethyl and Isopropyl Esters. The acid (6 mg, methacrylic or ethacrylic) was mixed with 2-phenylethyl or isopropyl alcohol (1 eq.), dicyclohexylcarbodiimide (1 eq.), and a catalytic amount of 4-dimethylaminopyridine in dichloromethane. The mixture was stirred overnight and flashed through a silica gel column with hexane:diethyl ether (6:4). *Isopropyl methacrylate*: EIMS m/z: 128 [M]$^+$ (4), 113 (8), 88 (2), 87 (54), 86 (5), 83 (7), 82 (3), 74 (2), 70 (13), 69 (99), 59 (28), 58 (2), 45 (7), 44 (2), 43 (84), 42 (18), 41 (100), 39 (51), 38 (6), 37 (3). *Isopropyl ethacrylate*: EIMS m/z: 142[M]$^+$ (2), 109 (2), 102 (2), 101 (33), 104 (44), 97 (2), 85 (14), 84 (9), 83 (78), 82 (17), 81 (2), 73 (2), 69 (4), 59 (21), 58 (2), 57 (7), 56 (10), 55 (84), 54 (11), 53 (11), 52 (2), 51 (3), 50 (2), 45 (6), 44 (4), 43 (100), 42 (13), 41 (39), 39 (33), 38 (3). *2-Phenylethyl methacrylate*: EIMS m/z: 105 (11), 104 (100), 103 (5), 91 (8), 79 (3), 78 (5), 77(5), 69 (32), 65 (5), 63 (2), 51 (4), 41 (33), 40 (3), 39 (13). *2-Phenylethyl ethacrylate*: EIMS m/z: 105 (14), 104 (100), 103 (5), 91 (6), 83 (16), 79 (3), 78 (5), 77 (6), 65 (4), 56 (2), 55 (31), 54 (2), 53 (4), 51 (4), 39 (9).

Determination of Position of Double Bonds. Glandular extracts were methylthiolated according to Francis and Veland (1981). The adducts were extracted into dichloromethane and subjected to GC-MS analysis [Shimadzu GCMS-QP5050A fitted with an XTI-5-coated (0.25 μm) fused-silica capillary column (30 m $\times$ 0.25 mm)]. The oven temperature was held at 40°C for 3 min, increased at 15°C/min to 280°C, and then held for 15 min.

RESULTS AND DISCUSSION

GC-MS analysis of the defensive fluid of *T. slevini* indicated the secretion to be a mixture of low molecular weight carboxylic acids and hydrocarbons (Figure 1, Table 1), a combination frequently encountered in carabid beetles.

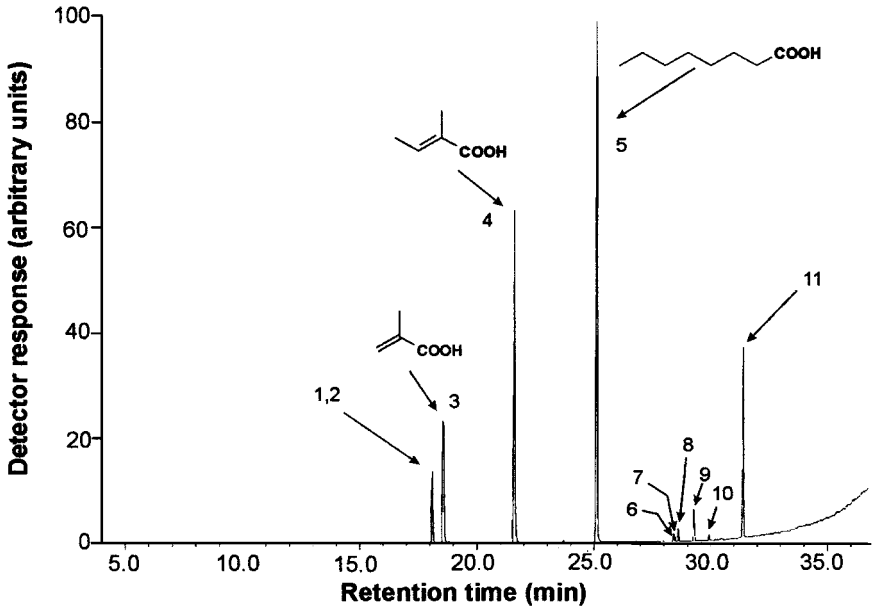


FIG. 1. Reconstructed gas chromatogram obtained from GC-MS analysis of a dichloromethane extract of the defensive secretion of *Trachypachus slevini* (see Table 1 for peak identifications). A fused-silica column (30 m × 0.25 mm) coated with ZB-FFAP was used. The oven temperature was kept at 40°C for 3 min and raised 6°C/min to 265°C.

Major constituents were identified by comparing their mass spectra and gas chromatographic retention times with those of authentic compounds. The major polar components in the fluid were identified as methacrylic and tiglic acids. However, the most abundant constituent was octanoic acid (caprylic acid). Although this is a carboxylic acid, the long alkyl chain renders it relatively nonpolar. Octanoic acid is not common in arthropods, but is known as a secondary or minor component of the secretions of the whip scorpion, *Mastigoproctus giganteus* (Lucas) (Eisner et al., 1961) and some darkling beetles (Tenebrionidae) (Tschinkel, 1975), which use a similar system for defense. In the case of *M. giganteus*, individuals spray a stream of acetic acid containing 5% octanoic acid. Many arthropods combine a polar irritant with nonpolar compounds to afford a more potent defensive mixture. The most likely role of nonpolar compounds is the transport of polar toxins, allowing penetration of the waxy epicuticle of arthropod predators (Eisner et al., 1961). It is likely that octanoic acid functions the same way in *T. slevini*, in relation to the polar components methacrylic and tiglic acids, as it does in *M. giganteus* for acetic acid.

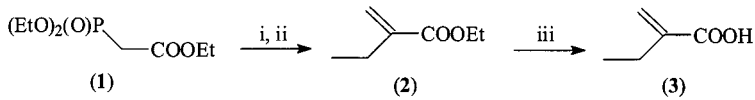
TABLE 1. VOLATILE COMPOUNDS CHARACTERIZED FROM THE PYGIDIAL GLAND CONTENTS OF *Trachypachus slevini*

Peak No.[a]	Compound	Relative amount[b]
1	isovaleric acid	12
2	2-methylbutyric acid	2
3	methacrylic acid	28
4	tiglic acid	68
5	octanoic acid (caprylic acid)	100
6	nonanoic acid	2
7	(*Z*)-9-tricosene[c]	1
8	(*Z*)-7-tricosene[c]	3
9	unidentified	7
10	unidentified	1
11	(*Z*)-9-pentacosene[c]	37

[a] Peak numbers refer to chromatographic peaks in Figure 1.
[b] There was no qualitative variation, however, some quantitative variation in the composition was observed. The values listed here pertain to one sample. The given amounts of components are relative to octanoic acid, which is listed as 100.
[c] Gas chromatographic retention times and corresponding infrared spectra indicate the configuration of the double bond to be *cis*.

In addition, the pygidial gland contents of *T. slevini* show several unsaturated hydrocarbons such as (*Z*)-9-pentacosene. Such long-chain hydrocarbons have been reported from secretions of Hymenoptera (Calam, 1969) but not from beetles. The longest unsaturated hydrocarbon previously reported from beetles is 1-nonadecene (Tschinkel, 1975).

The pygidial gland chemistry of *T. gibbsii* is unique in having the combination of methacrylic and ethacrylic acids as its two major components (Figure 2, Table 2). The later has been reported only rarely from arthropod secretions (Waterhouse and Wallbank, 1967; Moore and Wallbank, 1968; Benn et al., 1973). Identification of ethacrylic acid solely on the basis of its EI mass spectrum is not straightforward since many monounsaturated acids of molecular formula $C_5H_8O_2$ that have been characterized from arthropod secretions produce similar spectra. In order to confirm its identification, and those of a few other compounds that appeared to be esters of ethacrylic acid, we undertook the synthesis of ethacrylic acid according to the reaction steps illustrated in Scheme 1.



(i) NaH, EtI, THF, room temperature; (ii) NaH, $(CH_2O)_n$, Et_2O, 0–20°C, 1.5 hr; (iii) NaOH, H_2O, MeOH, 100°C, 4.5 hr.

SCHEME 1.

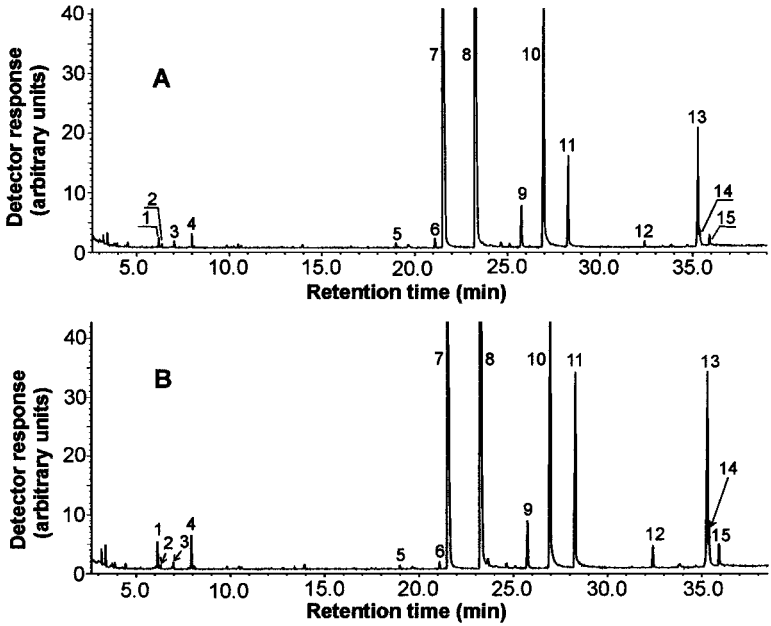


FIG. 2. A. Reconstructed gas chromatogram obtained from a glandular extract of *Trachypachus gibbsii female* by GC-MS analysis. B. Reconstructed gas chromatogram obtained from a glandular extract of *Trachypachus gibbsii male* by GC-MS analysis.

Alkylation of triethyl phosphonoacetate (**1**) with ethyl iodide in the presence of a base, followed by the Horner-Wadsworth-Emmons reaction with paraformaldehyde, gave ethyl 2-ethylacrylate (**2**) (Wadsworth and Emmons, 1961; Jew et al., 1997). Subsequent hydrolysis of **2** gave ethacrylic acid (**3**). The EI mass spectrum and GC retention time of the synthetic product on an FFAP column were indistinguishable from those recorded from the natural product. Moreover, this identification was confirmed by the GC-IR studies (Figure 3).

Similarly, the isopropyl and phenylethyl esters of methacrylic and ethacrylic acids were synthesized and their properties were compared with those recorded for the natural materials. To the best of our knowledge, isopropyl methacrylate, isopropyl ethacrylate, phenethyl methacrylate, and phenethyl ethacrylate have not been previously identified as secondary metabolites from any source.

Several unsaturated hydrocarbons were found in the pygidial gland fluids of *T. slevini* and *T. gibbsii*. Iodine-catalyzed addition of dimethyl disulfide (DMDS) followed by GC-MS analysis of the adducts was carried out (Francis and Veland, 1981). GC-MS analysis of glandular extracts of *T. gibbsii* showed the presence of two pentacosenes and a tricosene. The two adducts derived from pentacosenes showed their molecular ion at m/z 444. The first-eluting isomer showed two peaks

TABLE 2. VOLATILE COMPOUNDS CHARACTERIZED FROM THE PYGIDIAL GLAND CONTENTS OF *Trachypachus gibbsii*

Peak No.[a]	Compound	Relative amount[b]	
		Female	Male
1	isopropyl methacrylate	1	3
2	3-hexanone	trace	trace
3	2-hexanone	1	1
4	isopropyl ethacrylate	1	3
5	isobutyric acid	1	trace
6	2-methylbutyric acid	1	1
7	methacrylic acid	85	73
8	ethacrylic acid (**3**)	100	100
9	2-phenylethanol	7	7
10	2-phenylethyl methacrylate	49	50
11	2-phenylethyl ethacrylate	14	33
12	(*Z*)-7-tricosene[c]	1	3
13	(*Z*)-9- pentacosene[c]	16	31
14	(*Z*)-7-pentacosene[c]	2	7
15	a pentacosadiene[d]	1	3

[a] Peak numbers refer to those given in Figure 2a and 2b.
[b] One sample per sex analyzed.
[c] Gas chromatographic retention times and corresponding infrared spectra indicate the configuration of the double bond is *cis*.
[d] Position and configuration of the double bonds undetermined.

at m/z 271 and 173, while the later-elution isomer showed signals at m/z 299 and 145 indicating the compounds to be 9- and 7-pentacosene, respectively. According to the intensities, the 9-pentacosene is the more abundant isomer. Similarly, the adduct derived from tricosene gave the molecular ion at m/z 416 and fragments at m/z 271 and 145 indicating a double bond at position 7. The two tricosenes from glandular extracts of *T. slevini* were found to be 7- and 9-tricosene, with 7-tricosene the more abundant isomer. The pentacosene was characterized as 9-pentacosene.

Both *T. slevini* and *T. gibbsii* have major compounds that are identical, or biosynthetically similar to, those found in the majority of carabids, and can be grouped within the same chemical classes used by Moore (1979) for Carabidae. *Trachypachus* shares only octanoic and nonanoic acids with some Hydradephaga. These compounds are rarely found in aquatic beetles, and such occurrences are presumed to be derived within dytiscids [e.g., Colymbetinae: *Platambus maculatus* (L.), *Ilybius ater* (Degeer); Laccophilinae: *Laccophilus minutus* (L.)] (Dettner, 1985). While this might provide some evidence that trachypachids fall within Dytiscidae, it does not corroborate morphological evidence that trachypachids are related to hydradephagans, as that evidence clearly suggests trachypachids

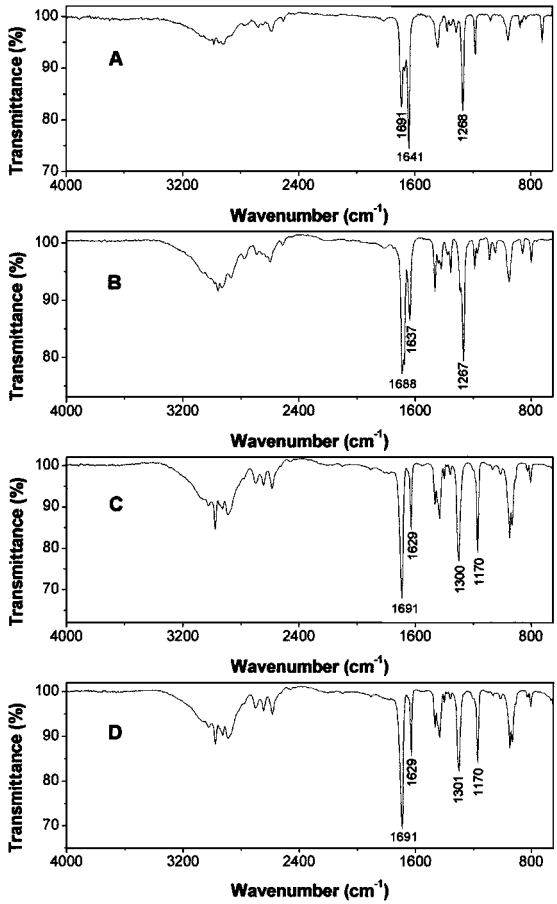


FIG. 3. Condense-phase FTIR spectra recorded by GC-FTIR spectrometry of authentic senecioic acid (A), angelic acid (B), and ethacrylic acid (C), and that corresponding to peak No. 8 (Figure 2) of the chromatogram recorded from the *Trachypachus gibbsii* defensive secretion (D). Resolution = 8 cm^{-1}.

are outside of the family Dytiscidae (Bell, 1966; Hammond, 1979; Roughley, 1981).

A third species, *Trachypachus holmbergi* Mannerheim, is still under study, but preliminary results indicate that the well-known terpene aldehyde citral, and an unidentified sulfur compound are present. Both of these are distinct from compounds found in the two species discussed here. Sulfur compounds are known from a scattering of adephagan taxa [e.g., *Amphizoa* (Dettner, 1990) and various carabids (unpublished data)]. Citral is not known in adephagan beetles but is

reported from Staphylinidae (Blum, 1981). Citral is biosynthetically distant from the aromatic aldehydes (e.g., *p*-hydroxybenzaldehyde) that are commonly found in hydradephagan species.

On the basis of our analyses, each of the three *Trachypachus* species is distinctive in its defensive chemical composition. However, *T. slevini* and *T. gibbsii* both use unsaturated and saturated carboxylic acids in conjunction with hydrocarbons. Ecologically, they all appear to be similar. All three North American species are decidedly terrestrial, found burrowing through loose soil and pine duff (Shull et al., 2001). Given that there is no obvious ecological differentiation in the genus and that the fossil record indicates that this is an ancient group, two scenarios can account for the chemical differentiation. Either the rate of change in the chemical constituents for *Trachypachus* species is relatively high or the divergence of their most recent common ancestor is ancient.

There is no evidence that the rate of character change in general for *Trachypachus* species is elevated as compared to ecologically similar species of Carabidae. The four *Trachypachus* species are morphologically distinct (body form and genitalia) but not exceptionally different. The relative morphological difference is typical, or even somewhat less, from what is commonly recognized between species within carabid genera such as *Bembidion* or *Pterostichus*.

The age of divergence between *Trachypachus* species can be placed in relative terms and broadly estimated using defensive chemical data. In carabid groups with reasonably densely sampled species-level defensive chemical data, those thought to be relatively recent radiations (e.g., *Pterostichus*, Harpalini, Platynini) are remarkably uniform in composition (Dazzini-Valcurone and Pavan, 1980; Will et al., 2000). Older, relictual groups (Erwin, 1985) [e.g., Carabini (Dazzini-Valcurone and Pavan, 1980)] and Gondwanian age groups [e.g., Loxandrini, Pterostichini auctorum (Will, 2000; Will et al., 2000)] generally show clear chemical differentiation. Chemical differences in extant species of *Trachypachus* are consistent with a divergence from their common ancestor during the Cretaceous, somewhere between 125 mya (separation of Australia, India, and Antarctica from Africa and South America) and 50 mya in the Eocene (proposed timing of transberingial dispersal of *Trachypachus*) (Erwin, 1985).

Even though each of the species is distinct chemically, the classes of compounds in *Trachypachus* are more similar to those known from Carabidae than from Hydradephaga. As it is not yet clear whether these similarities are apomorphic or plesiomorphic, we cannot resolve the relationship of trachypachids with other adephagan families on the basis of this evidence. However, on chemical grounds alone, it does allow the possibility that *Trachypachus* is more closely related to Carabidae than to any hydradephagan family. As suggested by Moore (1979), the defensive chemical evidence must be combined with the growing body of data for Adephaga to determine the most likely set of relationships for the included taxa.

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A FLAVANONE AND TWO PHENOLIC ACIDS FROM *Chrysanthemum morifolium* WITH PHYTOTOXIC AND INSECT GROWTH REGULATING ACTIVITY

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Abstract—Leaves of *Chrysanthemum morifolium* cv. Ramat were extracted sequentially with hexane, ethyl acetate, and methanol. The methanol fraction, when incorporated into artificial diet was found to reduce the growth of cabbage looper (*Trichoplusia ni* Hubner) larvae at concentrations between 500 and 5000 ppm of diet. Fractionation of the methanol extract on a Sephadex column yielded five fractions, three of which reduced the weight of larvae relative to the control. One fraction was analyzed using high performance liquid chromatography (HPLC) and found to contain three main constituents. These compounds were purified using a combination of gel permeation chromatography on Sephadex LH20 and HPLC, and analyzed by ^{1}H and ^{13}C NMR as well as undergoing chemical and physical analyses. The compounds were identified as: **1**, chlorogenic acid (5-*O*-caffeoylquinic acid); **2**, 3,5-*O*-dicaffeoylquinic acid; and **3**, 3′, 4′, 5-trihydroxyflavanone 7-*O*-glucuronide (eriodictyol 7-*O*-glucuronide). At concentrations between 100 to 1000 ppm these compounds reduced both growth and photosynthesis of *Lemna gibba* L. with the order of efficacy being: flavanone > chlorogenic acid > 3,5-*O*-dicaffeoylquinic acid. Furthermore, when

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incorporated separately into artificial diet these compounds, at 10 to 1000 ppm, enhanced or reduced growth of the cabbage looper (*Trichoplusia ni*) and gypsy moth (*Lymantria dispar* L.) .

Key Words—*Chrysanthemum morifolium*, flavanone, phenolics, phenolic acids, cabbage looper, gypsy moth, *Lemna gibba*.

INTRODUCTION

Phenolics are a large class of plant secondary metabolites that include flavonoids and various phenolic acids. Subsequent to Elliger et al. (1980), there has been increased interest in the function of these compounds within plants, and their interaction with other organisms and environmental factors. For example, flavonoids are larval growth inhibitors of the European corn borer *Ostrinia nubilalis* Hubner (Abou-Zaid et al., 1993), gypsy moth *Lymantria dispar* (L.) (Beninger and Abou-Zaid, 1997), and forest tent caterpillar *Malacosoma disstria* Hubner. (Abou-Zaid et al., 2000). In addition, flavonoids provide plants with color, UV-B protection, and are important in preventing microbial invasion (Harborne, 1994; Harborne and Williams, 2000).

Plant-derived allelochemicals or secondary metabolites, many of which are phenolics, may limit the host range of phytophagous insects and prevent growth of competing vegetation based on their activity as deterrents and toxins. These compounds act as a plant's primary defense against herbivory, but also have a number of other functions within the plant (Harborne, 1994). Species of *Chrysanthemum* produce a number of biologically active compounds. For example, pyrethrin is a compound derived from *Chrysanthemum cinerariefolium*, also known as the pyrethrum flower (Barthomeuf et al., 1996). There are six related insecticidal compounds that occur naturally in the crude pyrethrum flower material. We chose to examine the secondary chemistry of *Chrysanthemum morifolium* c.v. Ramat since this plant is also known to produce allelochemicals (Kil and Lee, 1987) and flavonoids of medicinal value (Hu et al., 1994).

The objective was to isolate and identify phenolics from *C. morifolium* and to determine their biological activity using two generalist insect species, gypsy moth and cabbage looper, and one higher plant species, *Lemna gibba*. Cabbage loopers feed on a wide variety of plants, and larvae have been found on *C. morifolium* plants (Soo Hoo et al., 1984), while the gypsy moth is a serious defoliator of forest trees in North America (Beninger and Abou-Zaid, 1997). *Lemna* spp. are small aquatic plants that are useful for the assessment of potential allelochemicals due to their ease of culture, high capacity for the assimilation of foreign chemicals (Greenberg et al., 1992), and also for the small amounts of compounds that are needed in the assay (Einhellig et al., 1985).

METHODS AND MATERIALS

Plants and Extraction Procedure. Foliage from greenhouse grown *Chrysanthemum morifolium* cv. Ramat was collected in July and August of 2001, stored frozen at −80°C, and lyophilized to dryness. Dried leaf material was ground to a fine powder in a blender and loaded onto a glass column (55.0 × 10.0 cm) fitted with a Teflon stopcock. Powdered leaf material (500 g) was extracted with 100% hexane, 100% ethyl acetate, and finally with 100% methanol until the solvents ran clear (i.e., no visual color). Extracts were evaporated to dryness at 45°C under reduced pressure. The hexane and ethyl acetate fractions were recovered in a minimum amount (4.0 ml) of solvent and dried under nitrogen. The methanol extract was recovered in 100 ml methanol:water 1:1 (v:v), placed into a −80°C freezer overnight, freeze dried until in powder form, and stored at −20°C.

Isolation of Secondary Metabolites. Two gram aliquots of the dried methanol extract were dissolved in 4.0 ml methanol:water 4:1 (v:v) and loaded onto a column (25 × 6 cm) containing 130 g of Sephadex LH20 that had been equilibrated with 100% methanol. Separation of the extract was monitored with a hand-held ultraviolet (UV) lamp and fractions (i.e., bands) were collected based on visual differences in absorbance at 364 nm. Recovered fractions were evaporated to dryness at 45°C under reduced pressure, recovered in 4.0 ml methanol:water 8:2 (v:v), frozen, and freeze dried as above. Fraction 4 was further separated on a 25 × 2 cm column containing 6.0 g Sephadex LH20 previously equilibrated in methanol:water 8:2 (v:v) and was eluted with the same solvent. Final purification of compounds was achieved on an Agilent model 1100 HPLC equipped with a degasser, quad pump, autosampler, and diode array detector (DAD). Samples were detected at 264 nm, and spectra of peaks were scanned between 200 and 550 nm. The column used was a CapCell Pak AG 120 C18 (4.6 × 250 mm, 5 μ particle size). The solvent system was described by Abou-Zaid et al. (2000) and is as follows: solvent A = 5% aqueous formic acid; solvent B = acetonitrile/methanol 5/95; elution steps: 0–3 min isocratic (85% A:15% B); 3–8 min, gradient (85% A:15% B–76% A:24% B); 8–11 min, isocratic (76% A:24% B); 11–18 min, gradient (76% A:24% B–66% A:34% B); 18–28 min, gradient (66% A:34% B–54% A:46% B); 28–39 min, gradient (54% A:46% B–5% A:95% B) 39–42 min, isocratic (5% A:95% B); 42–47 min, gradient (5% A:95% B–85% A:15% B); 47–55 min, isocratic (85% A:15% B). The flow rate was set to 0.9 ml/min.

Spectral Determination of Isolated Compounds

Ultraviolet. The UV spectra between 200 and 550 nm were recorded for each compound with a scan from 200 to 550 nm using the HPLC DAD and the

solvent system indicated above. Using the above solvent system, a 10 μl injection of fraction 4 at a concentration of 1.0 mg/ml was made.

Nuclear Magnetic Resonance Spectroscopy (NMR). Compounds **1** (20 mg), **2** (7 mg), and **3** (10 mg) were dissolved in 1.0 ml of deuterated dimethylsulfoxide (DMSO-$_{d6}$) and placed into NMR tubes. Spectra were recorded on either a Bruker 400 or 600 MHz NMR with X-WIN software at the University of Guelph NMR facility. A 5-mm multinuclear probe set to 35°C was used to obtain the spectra.

Mass Spectrometry. ESI-MS was performed on compounds **2** and **3** with a Micromass Quatro II triple quadrupole mass spectrometer. The sample was acquired in negative ion mode (cone voltage = 20–40 V) using flow injection analysis in methanol:water 1:1. The mass spectrometer was located at the University of Western Ontario, London, Ontario, Canada.

*Acid Hydrolysis of Compound **3**.* Hydrolysis of the glycoside followed the procedure of Markham (1982). Compound **3** (3.6 mg) was dissolved in 5.0 ml of 2 N HCl:MeOH 1:1 (v:v), heated in a hot water bath (100°C) under a cooling condenser for 60 min, and then evaporated to dryness under vacuum at 40°C . The dried sample was suspended in 5.0 ml of MeOH:H_2O (1:1) and subjected to HPLC. After HPLC analysis, the sample was evaporated to dryness, redissolved in 5.0 ml HCl:MeOH 1:1 (v:v), and subjected to two more hours of acid hydrolysis following the procedure described above. This was done to determine whether complete hydrolysis would occur after a longer period of time. After drying, resuspension, and HPLC, **3** was dried, extracted with 5.0 ml of ethyl acetate:H_2O 3:2 (v:v), and shaken vigorously in a test tube. The ethyl acetate and water fractions were decanted and analyzed by HPLC.

Insect Bioassays. First or second instar gypsy moths and cabbage looper were obtained from the insect rearing unit at the Canadian Forest Service, Great Lakes Forestry Centre, Sault Ste. Marie, Ontario, Canada. If obtained as first instars, the insects were reared to the second instar on control diet prior to bioassay. Extracts, fractions, or pure compounds were dissolved in acetone:water (1:1, v:v) and added to artificial diet as it cooled to 50°C during preparation (Abou-Zaid et al., 1993; Beninger and Abou-Zaid, 1997). Artificial diets for cabbage looper and gypsy moth were obtained from BioServe® (Frenchtown, NJ, USA). Second instars were placed onto control or treated diet plugs in Solo® cups (one larva per cup and 40 larvae per treatment), and larval weight was recorded at 48 hr intervals. Larvae were reared in an environmental chamber under full spectrum light (16:8 L:D) at 28°C. Chlorogenic acid is available commercially at minimal cost and bioassays were conducted at concentrations of 100 and 1000 ppm until pupation. However, for 3,5-*O*-dicaffeoylquinic acid and eriodictyol 7-*O*-glucuronide, quantities were limited to the amount that could be isolated from the plant. Bioassays of these compounds were conducted at 10 and 100 ppm and terminated when diet containing the compound was exhausted.

Lemna gibba Assay. Cultures were maintained on Hutner's medium with 1% sucrose added to water following the method of Greenberg et al. (1992). One to two weeks prior to bioassay, *L. gibba* was removed and transferred to 2800 ml glass erlenmeyer flasks containing water to which only Hutner's medium was added. Test compounds were dissolved in water and diluted to the appropriate concentration in Hutner's medium. Single *L. gibba* plants (three- to four-frond stage of development) were placed into each of six wells (one plant per well). Each control and treatment consisted of six replicates (i.e., six plants with one plant per well). Concentrations of compounds used for the treatments were 100 and 1000 ppm, respectively. Sterile Corning Inc. Costar® 24-well-culture plates that, except for the interior of the wells and cover, had been painted black were used for the bioassays. Measurements of the photosynthesis parameters F_v/F_m and F_v/F_m total photosynthetic area were taken daily using a fluoroimager. Experiments and *L. gibba* cultures were maintained in an environmental chamber under constant full spectrum light at 25°C.

Fluoroimager. A Model FISY5006 fluoroimager (Technologia Ltd., John Tabor Labs, University of Essex, C04 3SQ UK) was used. The parameter measured to determine the efficiency of photosynthesis is F_v/F_m, which is the maximum quantum yield of photosystem II ($F_v = F_m - F_0$ where F_m = maximum fluorescence in the dark adapted state and F_0 = minimum level of fluorescence in the dark adapted state) (Maxwell and Johnson, 2000). Cell-culture plates with *L.gibba* plants in the wells were placed into the dark for 30 min before recording F_0 using a measuring pulse of 0.32 μmol m^{-2} sec^{-1} photosynthetically active photon flux density (PPFD) and F_m using a saturating flash of 2800 μmol m^{-2} sec^{-1} PPFD.

Statistical Analyses. The SAS® (1989) general linear model (GLM) procedure was used to perform one-way ANOVAs followed by Student–Newman Keuls comparisons of means to determine differences between treatments for both the *L. gibba* and insect assays. Wherever the phrase "significantly different" is used it indicates that the differences between treatment groups were significant at $P < 0.05$.

RESULTS

Cabbage Looper. Cabbage looper larvae were used to determine whether the crude methanol extract had an effect on growth and development and, thus, showed signs of biological activity. At concentrations of 500–5000 ppm, the methanol extract significantly reduced growth of cabbage looper larvae after six days of feeding (Figure 1A). After fractionation of the methanolic extract, it was determined that fractions 3, 4, and 5 fed at 1000 ppm reduced growth of the looper larvae (Figure 1B). Furthermore, the main components found in each of these fractions were chlorogenic acid, 3,5-dicaffeoylquinic acid, and eriodictyol 7-*O*-glucuronide.

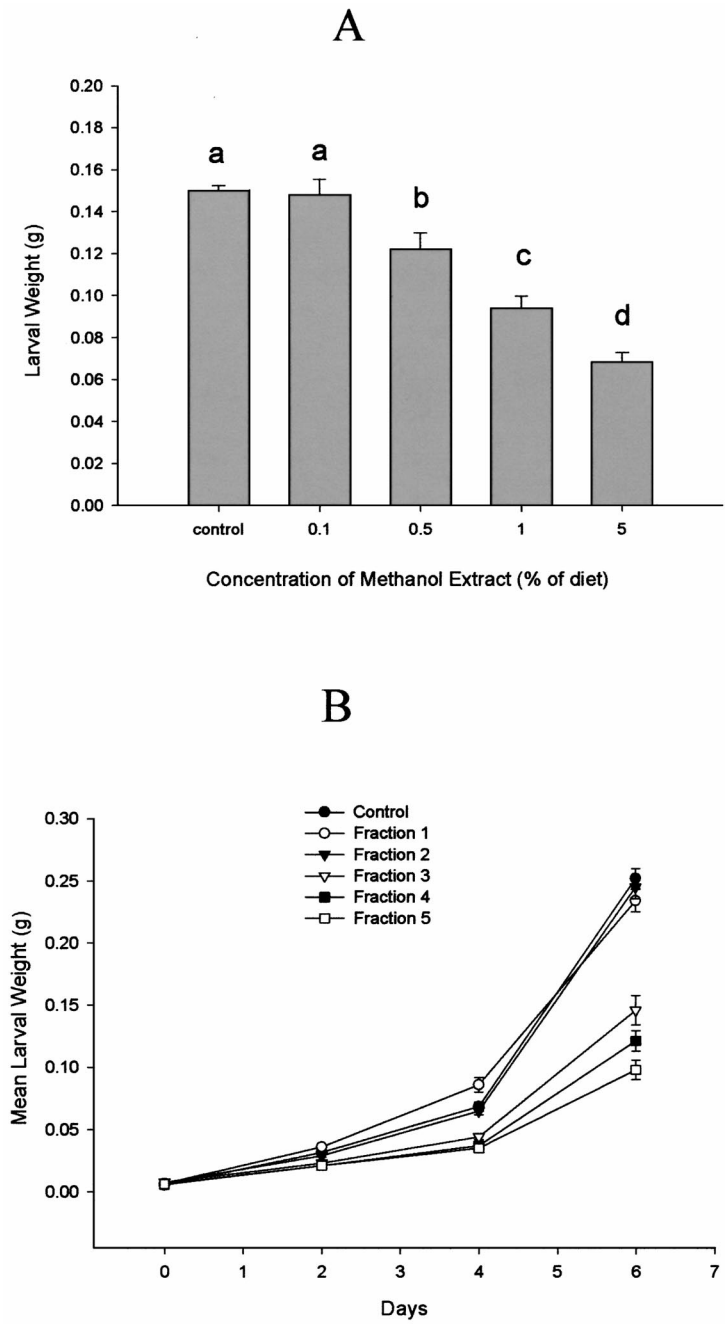

FIG. 1. (A) Effect of various concentrations of *C. morifolium* methanolic extract on growth of cabbage looper after 6 d of feeding. Means followed by the same letter are not significantly different. (B) Effect of different *C. morifolium* fractions on growth of cabbage looper. Fractions tested at a concentration of 0.1% in artificial diet.

Identification of Purified Compounds

Ultraviolet. UV spectra of **1** and **2** were identical with a peak maximum of 325 nm and a shoulder at approximately 300 nm (spectra not shown). This absorbance is characteristic of chlorogenic acid and its derivatives. The peak maximum for compound **3** was at 280 nm with a shoulder at 330–340 nm. This agrees with the published UV spectrum for the general class of flavonoids known as flavanones (Mabry et al., 1970).

Acid Hydrolysis. Prior to hydrolysis, compound **3** gave a single peak with a retention time of 17.7 min. After 1 hr of acid hydrolysis, the peak at 17.7 min was still visible, but a new, larger peak appeared at 24.75 min indicating the presence of the aglycone. Upon a further 2 hr of hydrolysis, HPLC analysis showed the same two peaks at the same relative intensities. These results indicated that the sample would only undergo partial hydrolysis, which means that the sugar moiety is likely to be a glucuronide (Markham, 1982).

NMR. The numbering system for chlorogenic acid follows Stevenson et al. (1993), while for 3,5-dicaffeoylquinic acid the carbons of the first and second caffeoyl moiety are numbered 1′–9′ and 1″–9″, respectively; the quinic acid moiety is numbered 1–7 following Chuda et al. (1996) and IUPAC (1976) recommendations. The numbering system for compound **3** follows that of Markham (1982).

*Compound **1***: chlorogenic acid (5-caffeoylquinic acid) (Figure 2A), ^{1}H NMR: δ1.75–2.01 (4H, m, 2 X H2, 2 X H6), 3.40 (1H, d, $J = 7.34$ H4′), 3.80 (1H, s, H3′), 5.07 (1H, s, H5′), 6.15 (1H, d, $J = 15.9$, H8), 6.75 (1H, d, $J = 8.12$, H5), 6.95 (1H, d, $J = 10.16$, H6), 7.03 (1H, d, $J = 11.45$, H2), 7.45 (1H, d, $J = 7.29$, H7), ^{13}C NMR: 36.7 (C6), 37.3 (C2), 68.7 (C3), 71.0 (C4), 72.7 (C5), 72.9 (C1), 114.3 (C2′), 114.8 (C8′), 115.5 (C5′), 121.3 (C6′), 125.6 (C1′), 144.6 (C7′), 145.6 (C3′), 148.3 (C4′), 165.8 (C9′),175.2 (C7).

*Compound **2***: 3,5-dicaffeoylquinic acid (Figure 2B). ^{1}H NMR: δ1.93–1.98 (2H, m, 2 X H2), 2.10–2.15 (2H, m, 2 X H6), 3.83 (1H, s, H4), 5.12 (1H, td, $J =$ 4.34, H3), 5.33 (1H, d, $J = 4.72$, H5), 6.13 (1H, d, $J = 15.9$, H8′), 6.23 (1H, d, $J = 15.9$, H8″), 6.70 (1H, d, $J = 3.62$, H5′), 6.76 (1H, d, $J = 4.52$, H5″), 6.99 (2H, d, $J = 8.4$, H6′, H6″), 7.05 (2H, d, $J = 7.43$, H2′, H2″), 7.43 (1H, d, $J =$ 13.7, H7′), 7.46 (1H, d, $J = 13.7$, H7″); ^{13}C NMR: 34.6 (C6), 35.6 (C2), 67.5 (C3), 70.5 (C4), 70.8 (C5), 72.4 (C1), 114.1 (C2′, C2″), 114.7 (C8′), 114.8 (C8″), 115.68 (C5′), 115.74 (C5″), 121.0 (C6′), 121.2 (C6″), 125.5 (C1′), 125.6 (C1″), 144.6 (C7′), 145.0 (C7″), 145.5 (C3′, C3″), 148.1 (C4′), 148.3 (C4″), 165.5 (C9′), 165.9 (C9″), 175.1 (C7).

*Compound **3***: 3′,4′,5-trihydroxyflavanone-7-*O*-glucuronide (eriodictyol 7-*O*-glucuronide) (Figure 2C): ^{1}H NMR: δ 2.73 (1H, d, $J = 17.2$, H3eq) 3.21–3.25 (3H, m, H2″, H3″, H4″ glucuronic acid), 3.29 (1H, t, H3ax), 3.92 (1H, dd, $J = 14.0$, H5″), 5.08 (1H, dd, $J = 12.39$, H1″), 5.43 (1H, t, H2), 6.13 (1H, s, H8), 6.16 (1H, s, H6), 6.17 (2H, d, $J = 4.4$, H2′, H6′), 6.90 (1H, d, $J = 4.4$, H5′), 12.01 (1H, s, H5 [OH]); ^{13}C NMR: 42.6 (C3), 72.0 (C4″), 73.2 (C2″), 74.9 (C3″), 76.4

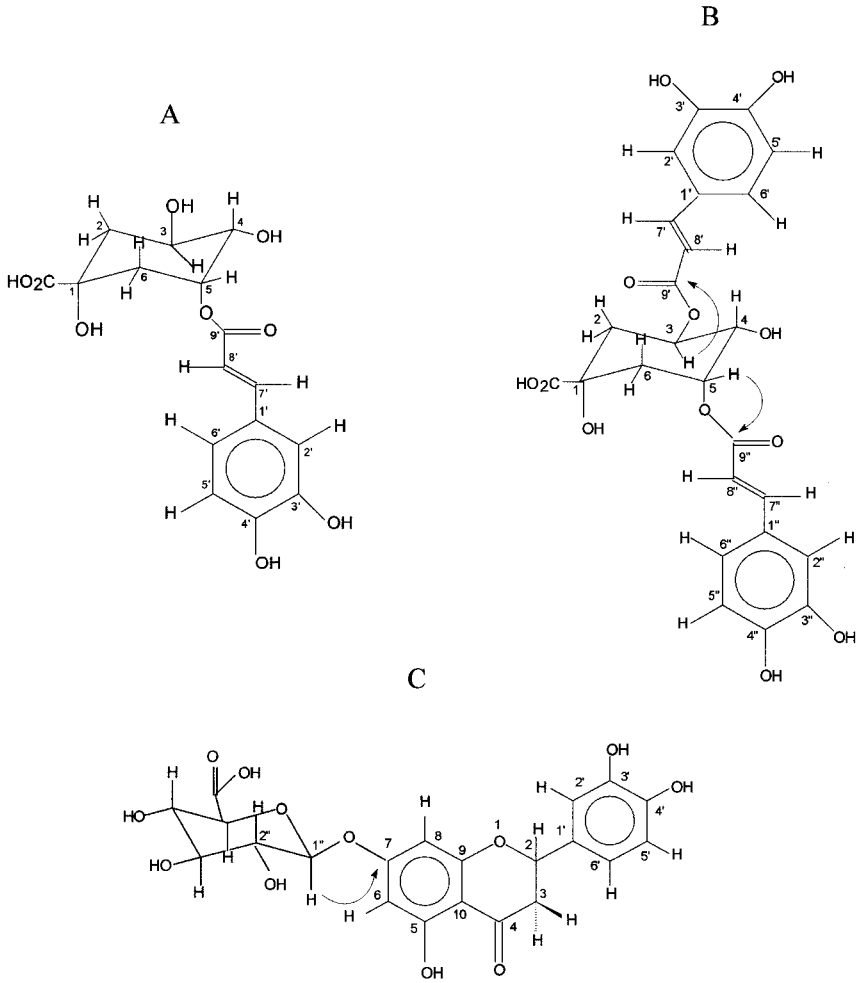

FIG. 2. Structures of compounds isolated from *C. morifolium.* (A) Chlorogenic acid; (B) 3,5-dicaffeoylquinic acid; and (C) eriodictyol 7-*O*-glucuronide. Arrows indicate HMBC correlations.

(C5″),79.1 (C2), 95.9 (C6), 96.9 (C8), 99.6 (C1″),103.7 (C10), 114.8 (C5′), 115.8 (C2′), 118.4 (C6′), 129.6 (C1′), 145.7 (C4′), 146.3 (C3′), 163.2 (C7), 165.5 (C9), 165.6 (C5), 171.4 (C6″), 197.6 (C4).

Compound **1** was subjected to HPLC analysis where a sample of fraction 4 at 1.0 mg/ml was spiked with 100 μl of chlorogenic acid standard at 1.0 mg/l. The peak increased in area while the UV spectrum remained the same. The UV spectrum of compound **2** was identical to that of chlorogenic acid; however, the HPLC

retention time differed and some of the proton signals were doubled. Therefore, this compound was determined to be a dimer with identical caffeoyl moieties attached to quinic acid. The above proton and carbon assignments were facilitated by 2D NMR techniques, such as a DQF-COSY, GHMQC, DEPT , GHMBC, and by comparison with published spectra of the same or similar compounds (Morishita et al., 1984; Agrawal and Bansal, 1989; Maruta et al., 1995; Chuda et al., 1996). The 3,5 configuration for **2** was confirmed from the GHMBC experiment that indicated secondary coupling of the 3 and 5 protons to the 9′ and 9″ carbons. The 7-attachment point of the glucuronic acid moiety for eriodictyol 7-*O*-glucuronide was also determined by examining the GHMBC spectrum and noting secondary coupling of the H1″ anomeric proton to C7.

Mass Spectrometry. Compound **2** gave a negative molecular ion peak $[M-H]^-$ at 515.49 m/z, which agrees with the molecular weight of 516.47 for 3,5-dicaffeoylquinic acid. Compound **3** gave a molecular ion peak $[M-H]^-$ at 463.38, which agrees with the molecular weight of 464.38 for eriodictyol 7-*O*-glucuronide. When the cone voltage was increased to 40 V for compound **3**, another fragment appeared at $[M-H]^-$ 287.25, which corresponds to the $[M-H]^-$ for the aglycone.

Lemna Assay. Chlorogenic acid was active at both concentrations, but the effects took somewhat longer to occur, so this particular experiment was 6 d in duration as opposed to 4 d for the other two compounds. At a concentration of 1000 ppm (2.8 mM), the F_v/F_m values were significantly reduced by day 1 and thereafter (Figure 3A). By day 6, the fronds were bleached and all photosynthesis terminated. There was no significant difference between the control and 100 ppm (0.28 mM) treatment (Figure 3A). The reduction in photosynthetic area (F_v/F_m) by 1000 ppm (2.8 mM) followed the same pattern as photosynthesis (Figure 4A). However, 100 ppm (0.28 mM) had a significant effect on F_v/F_m 6 d after treatment.

The compound 3,5-dicaffeoylquinic acid was the least active of the three compounds tested (Figures 3B and 4B). At both 1000 (2.2 mM) and 100 ppm (0.22 mM), there were significant differences when compared to the control on days 1, 2, and 3. However, by day 4 there were no significant differences in the F_v/F_m values for these concentrations (Figure 3B). Photosynthetic area was significantly reduced by 1000 ppm only at day 4 (Figure 4B). The highest concentrations of eriodictyol 7-*O*-glucuronide significantly reduced both F_v/F_m and photosynthetic area after 1 d, at which time all of the *L. gibba* fronds were completely bleached (Figures 3C and 4C). At the lower concentration, F_v/F_m was significantly lower than the control although this effect on photosynthetic area was not seen. Furthermore, the fronds were not completely bleached at 100 ppm.

Insect Assays

Cabbage Looper. Chlorogenic acid at both 100 (0.28 mM) and 1000 ppm (2.8 mM) significantly reduced larval weights by day 4 (Figure 5A). However,

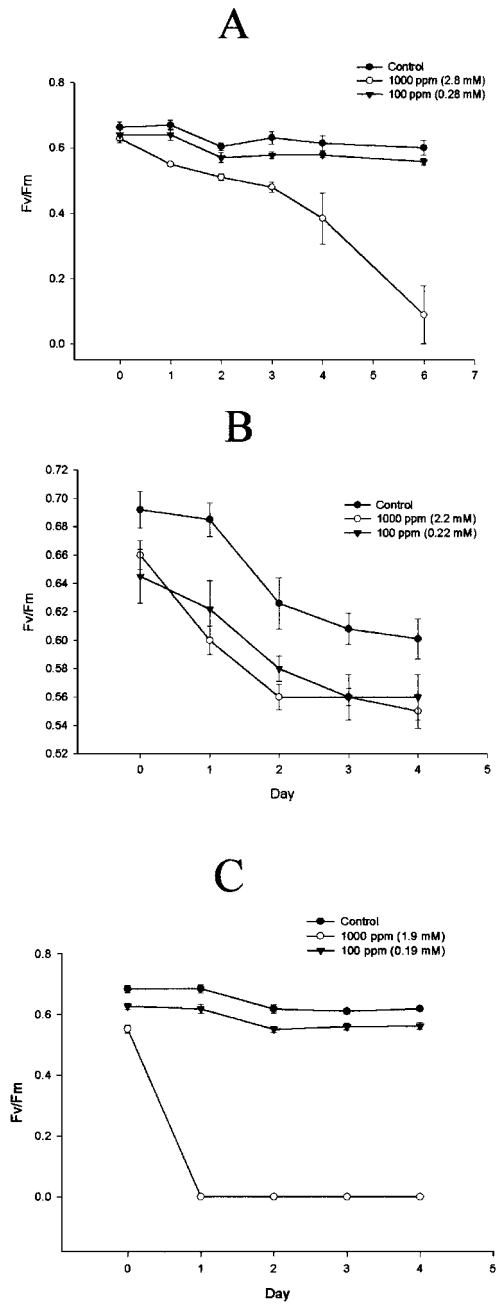

FIG. 3. Effect of isolated compounds on cmna photosynthesis (F_v/F_m). (A) chlorogenic acid; (b) 3,5-dicaffeoylquinic acid; and (c) eriodictyol 7-*O*-glucuronide.

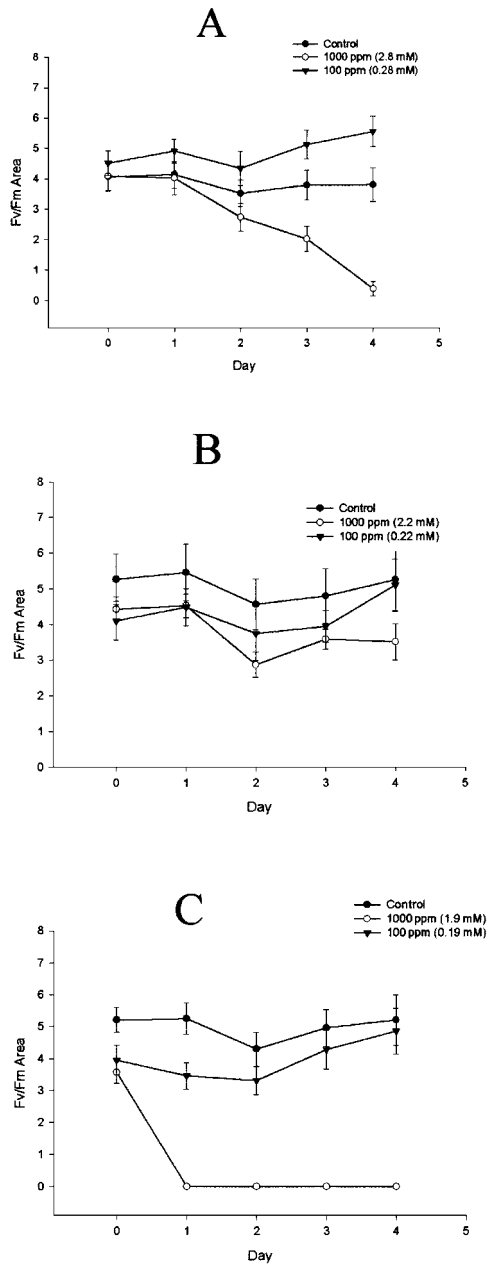

FIG. 4. Effect of isolated compounds on cmna net photosynthetic area (F_v/F_m area). (A) chlorogenic acid; (B) 3,5-dicaffeoylquinic acid; and (C) eriodictyol 7-*O*-glucuronide.

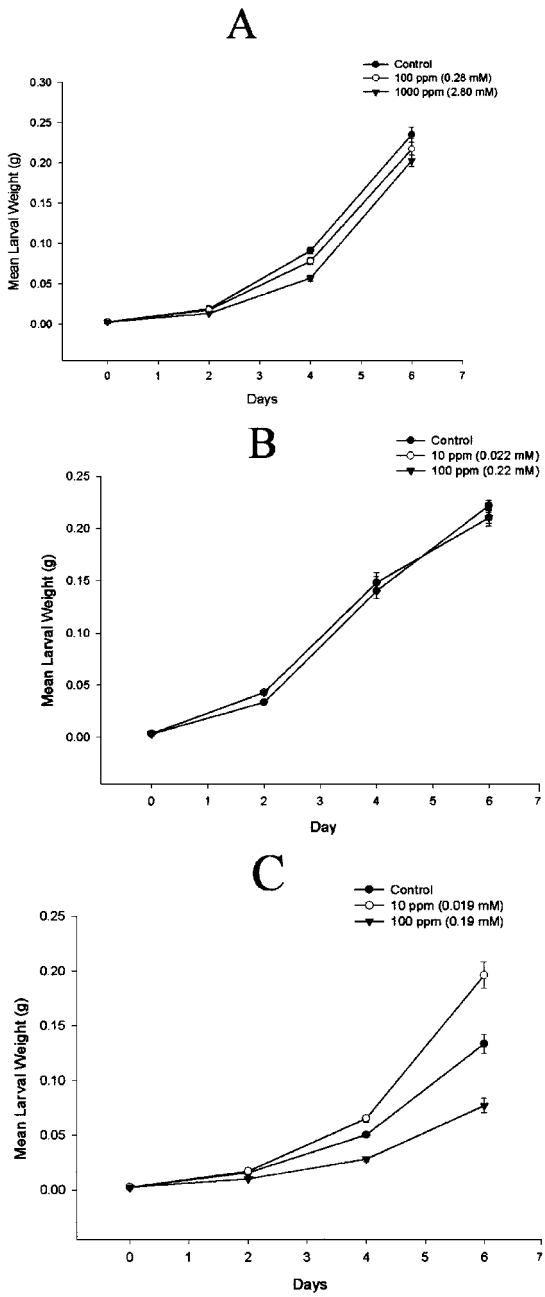

FIG. 5. Effect of (A) chlorogenic acid; (B) 3,5-dicaffeoylquinic acid; and (C) eriodictyol 7-*O*-glucuronide on growth of cabbage looper.

by day 6, only the weights of larvae feeding on diet containing chlorogenic acid at 1000 ppm, remained significantly different from the control. Regardless of the concentration of dicaffeoylquinic acid, there was no effect on growth relative to control (Figure 5B). Eriodictyol 7-*O*-glucuronide, increased (Figure 5C) larval weights at 10 ppm (0.019 mM) and reduced them at 100 ppm (0.19 mM).

Gypsy Moth. Concentrations of chlorogenic acid at both 100 and 1000 ppm significantly reduced growth of gypsy moth larvae from days 12 through 20 (Figure 6A). Little or no growth occurred after 20 d of feeding on chlorogenic acid at 1000 ppm. Dicaffeoylquinic acid at both concentrations significantly inhibited growth of gypsy moth by day 6 (Figure 6B), while eriodictyol-7-*O*-glucuronide at 100 ppm significantly reduced growth of gypsy moth by days 6 and 8. Conversely, 10 ppm eriodictyol 7-*O*-glucuronide significantly increased growth of gypsy moth by day 10 (Figure 6C).

DISCUSSION

We have identified for the first time in *C. morifolium* the flavanone eriodictyol-7-*O*-glucuronide that has both phytotoxic and insect growth regulatory activity. This is the first report of the phenolics chlorogenic acid and 3,5-dicaffeoylquinic acid being isolated from *C. morifolium*, although other types of phenolic acids (Kil and Lee, 1987) and sesquiterpenes (Osawa et al., 1973; Hu and Chen, 1997) have been found in this species. We also report for the first time on the phytotoxic action of 3,5-dicaffeoylquinic acid and eriodictyol 7-*O*-glucuronide.

At the end of six d, leaves of *L. gibba* were bleached by chlorogenic acid indicating loss of photosynthetic pigment. Although bleaching did not occur to the same extent with 3,5-dicaffeoylquinic acid, there was a significant reduction in photosynthesis. It is not clear whether reduction of photosynthesis and photosynthetic area by chlorogenic acid and its derivative, 3,5-dicaffeoylquinic acid, are due to direct or indirect effects on PS II. Phenolic acids can affect plant growth (and photosynthetic area) by interfering with either protein synthesis (Baziramakenga et al., 1997), or uptake of phosphorus, which, in turn, alters membrane potentials (Glass, 1973). Syringic, caffeic, and protocatechuic acids were found to reduce total chlorophyll and inhibit uptake of N, P, K, Fe, and Mo (Alsaadawi et al., 1986).

Of particular interest was the negative effect on photosynthesis and growth that the flavanone eriodyctiol had at concentrations of 100 (0.19 mM)–1000 ppm (1.9 mM). This is the first time a flavonoid from *C. morifolium* has been found to effect growth and photosynthesis of *L. gibba*. This compound is similar to baicelin, also isolated from *C. morifolium*, which has activity as an anti-HIV compound (Hu et al., 1994). In general, there is little information on how flavonoids with biological activity affect photosynthesis, either directly or indirectly. However, quercetin (3′,4′,5,7 tetrahydroxyflavonol) is known to inhibit mitochondrial ATPase (Lang

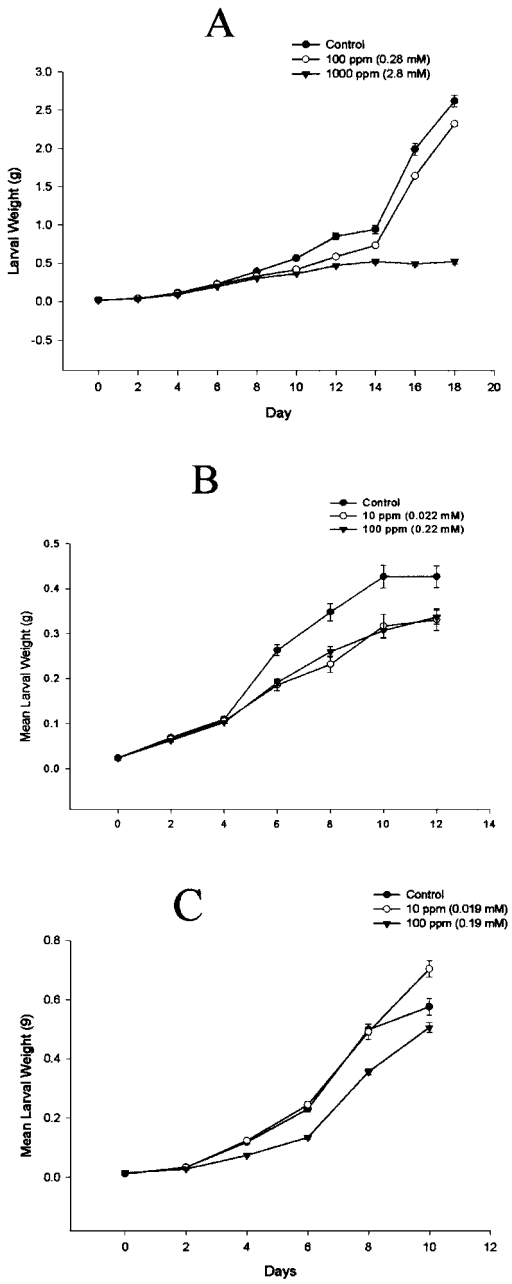

FIG. 6. Effect of (A) chlorogenic acid; (B) 3,5-dicaffeoylquinic acid; and (C) eriodictyol 7-*O*-glucuronide on growth of gypsy moth.

and Racker, 1974) and is also, along with other flavonoids, an inhibitor of cyclic AMP phosphodiesterase (Beretz et al., 1978). Therefore, the negative effects on photosynthesis observed for eriodictyol-7-*O*-glucuronide may be due to indirect effects on mitochondrial function.

The fact that phenolics and flavonoids do not act directly on either photosystem (PS) I or II may account for the relatively high concentrations needed to demonstrate phytotoxicity compared to synthetic PS II inhibitors. Atrazine, which binds to the $Q_B$site of PS II, is approximately 1000 times as active as any of the compounds found in *C. morifolium* ($pI_{50} = 6.1$–6.6, or 0.25 μM) (Fedtke, 1982). However, phenolics (which include flavonoids) have allelopathic properties (Kil and Lee, 1987), perhaps due to their build up to considerable concentrations in the soil matrix.

Chlorogenic acid had more of a negative effect on the growth and development of gypsy moth larvae than it did on cabbage looper (Figures 5A, 6A). Gypsy moth tends to be quite sensitive to phenolics in its diet, in particular at the early instars (Beninger and Abou-Zaid, 1997), whereas cabbage looper, which may encounter coumarins in its diet, has a more efficient detoxification mechanism for these types of compounds. Coumarins are known to induce glutathione transferase in cabbage looper (Yu, 1992). Levels of chlorogenic acid correlate with resistance to carrot fly (*Psila rosae* F.) (Ellis, 1999) and are also a factor in the resistance of corn to both fall armyworm (*Spodoptera frugiperda*) and corn earworm (*Helicoverpa zea*) (Gueldner et al., 1992). In addition, chlorogenic acid and its isomers inhibit growth and development of the tobacco armyworm (*Spodoptera litura* (Fab.)) (Stevenson et al., 1993). It is, therefore, not unexpected that it would inhibit growth of gypsy moth and cabbage looper.

At concentrations of 10 (0.216 mM) and 100 ppm (2.16 mM), 3,5-dicaffeoylquinic acid had no significant effect on cabbage looper, but both concentrations significantly reduced growth of gypsy moth. This is not surprising given the sensitivity of gypsy moth to phenolics discussed above. However, this compound was also the least active of the phenolics in the *Lemna* assays, which may mean that overall biological activity is quite low. Therefore, in general, dimers of chlorogenic acid may have reduced biological activity compared to monomers.

Of particular interest was the response of gypsy moth and cabbage looper to the flavanone eriodictyol 7-*O*-glucuronide. At 10 ppm (0.19 mM), it significantly enhanced growth of cabbage looper by day 4 (Figure 5C). However, at the higher concentration of 100 ppm (1.9 mM), it inhibited growth. This same effect was observed for gypsy moth, but did not take effect until day 10 (Figure 6C). Relatively little is known about how dietary flavonoids affect cabbage looper. For example, Sharma and Norris (1991) found that cabbage looper feeding on leaf disks treated with the isoflavanone daidzein and an unidentified flavonoid (X2) was reduced. The effect of quercetin on cabbage looper was investigated with regard to induction of antioxidant enzymatic responses (Ahmad and Pardini, 1990), but not its

effect on growth and survivorship. Similarly, Wheeler and Slansky (1991) tested crude extracts without attempting to isolate the active constituents from resistant and susceptible soybean cultivars. Hoffman-Campo et al. (2001) found that the flavonoid rutin negatively affected the survivorship of cabbage looper larvae at high concentrations and that at lower concentrations (e.g., 0.25% and 0.5%) pupal weight decreased. The current study is, to our knowledge, the first to fully characterize a flavanone glycoside and test its effects on larval growth of cabbage looper. This flavanone appears to be a feeding stimulant at low concentrations, but deleterious to larvae at higher concentrations in both gypsy moth and cabbage looper.

The roles that phenolics and flavonoids play in plant–plant, plant–microbe, and plant–insect interactions need further investigation. These compounds are ubiquitous in green plants yet their biological activity and functions within plants have received inadequate attention to date.

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TIMBER RATTLESNAKES (*Crotalus horridus*) USE CHEMICAL CUES TO SELECT AMBUSH SITES

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Abstract—Chemicals left by organisms moving through the environment are used by other organisms to mediate interspecific interactions. Most studies of chemical eavesdropping focus on prey responding to chemical cues from predators, despite the fact that chemical cues are frequently used by predators as a source of information about prey. *Crotalus horridus* uses a foraging strategy that is widespread among sedentary predators: the snake chooses a site where it is likely to encounter prey and remains immobile for many hours. I investigated this ambush hunting behavior in captive-raised timber rattlesnakes and provide evidence that sit-and-wait predators may discriminate among prey chemical cues, even when they have no prior experience with the prey. Snakes explored chemical cues with chemosensory behaviors, and more frequently adopted a stereotyped ambush foraging posture toward chemical cues from prey sympatric with their population of origin than either allopatric prey or sympatric nonprey species that are eaten by other viperids. These results support the notion that intra- and interspecific variation in diet may be mediated proximally by innate recognition of cues from particular prey items. This system also describes a bioassay that may be used in the isolation and identification of prey-derived kairomones. Studies such as this can be used to determine more realistic parameters for models of predator–prey interaction and foraging behavior that involve secretive, less active predators.

Key Words—Timber rattlesnake, *Crotalus horridus*, foraging behavior, chemical cues, prey discrimination.

INTRODUCTION

Organisms leave chemical cues on the substrate as they move through the environment. These are often used by other organisms to mediate interspecific

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interactions, providing prey information about their predators (Kats and Dill, 1998), and predators about their prey (Burghardt, 1990). Even though the use of chemical cues to find prey is a common hunting strategy, most work on chemical exploitation has focused on prey detecting predators (Kats and Dill, 1998).

Scent or odor trails left inadvertently by prey animals are generally assumed to be composed of chemicals released as an unavoidable consequence of excretory processes. These cues represent a reliable information source about potential prey, and are exploited by a variety of predators. Most studies of chemosensory prey recognition focus on actively foraging predators that use chemical cues to find and track prey (Burghardt, 1990; Stowe et al., 1995; Finelli et al., 2000; Koivula and Korpimaki, 2001). However, chemical cues are also used by sit-and-wait predators to locate profitable ambush sites at which to sit and wait for prey (Downes, 1999; Roth et al., 1999; Carroll, 2000; Persons and Rypstra, 2000). Many aspects of the use of prey chemical cues are likely to differ between ambush and active foragers, since they seek to derive different information.

For an ambush strategy to be effective, predators must be able to identify sites where they are likely to encounter their prey. The use of chemicals to select profitable ambush sites requires some means of identifying species-specific cues that have been left relatively recently. The extensive literature on actively foraging squamate reptiles indicates that many species are born with an ability to use chemical cues in discriminating among prey (Burghardt, 1990). These studies use similar methods to present predators with chemical cues (reviewed in Cooper, 1998): a cotton-tipped applicator is impregnated with chemicals from the integument of a potential prey species and then placed in the vicinity of the predator. The number of tongue-flicks (indicative of level of vomeronasal functioning) and the propensity to bite the applicator is then used as an index of positive responses. Studies following this methodology have found that actively foraging snakes and lizards discriminate among different prey chemical cues, and that these preferences vary geographically (Arnold, 1981). Presumably, this predisposition toward certain prey is an efficient means of focusing foraging behavior on prey that is beneficial, because it is either abundant, relatively easy to catch, or energy rich.

However, predators that typically do not actively track their prey fail to respond to chemical cues that are presented following the cotton applicator method. In particular, most iguanian and agamid lizards (Cooper, 1995) and viperid snakes (Chiszar and Scudder, 1980) exhibit no response to prey chemical cues presented on cotton-tipped applicators. It may be that for some cases the chemical cues were not presented in an ecologically relevant manner. Many snakes are primarily ambush predators, moving widely through the environment in search of chemical cues, which are then used in ambush site selection (reviewed in Greene, 1992). Prairie rattlesnakes (*Crotalus viridis*), pygmy rattlesnakes (*Sistrurus miliarius*), and broad-headed snakes (*Hoplocephalus bungaroides*) have all been shown to use chemical cues to locate suitable ambush sites (Duvall et al., 1990; Downes, 1999;

Roth et al., 1999; Theodoratus and Chiszar, 2000). Since chemosensory information is so important for snakes in general (Halpern and Kubie, 1984; Schwenk, 1995), the use of prey chemical cues exhibited by these species is probably characteristic of most snakes that ambush their prey.

In this study, I examine the selection of ambush sites by a sit-and-wait foraging viperid, the timber rattlesnake (*Crotalus horridus*), presented with chemical cues derived from various potential prey species. This study is the first to examine the ability of a sit-and-wait predator to discriminate among various chemical cues when selecting an ambush site.

METHODS AND MATERIALS

The snakes used in this experiment consisted of 24 *Crotalus horridus*, born in the laboratory to three wild-caught females that were caught on August 15, 1999, in Wyoming, Clinton, and Lycoming Counties, Pennsylvania. I conducted experiments between October 1, 2000, and May 1, 2001, during which the 24 snakes ranged from 45.5 to 66.7 cm total length and from 64 to 196 g in body mass. Snakes were housed individually in 20 gallon aquaria and reared on a diet of laboratory mice. The snakes were maintained in a Cornell University animal-holding facility at 22–26°C under a 12L:12D light cycle, with a water dish and heating pad situated at one end of each aquarium.

I tested the 24 experimental subjects once each on a series of aqueous extracts of the integument of 8 vertebrate species, some of which were known to be prey for *C. horridus* in at least some part of its range (Clark, 2002). Each subject was also tested using tap water as a control. All aqueous extracts were prepared by placing a living, intact animal into a water bath for 10 min in the proportion of 1 ml of water per gram of body mass. Extracts were refrigerated until use. All extracts were used within 8 hr.

I made extracts from green frog (*Rana clamitans*), great plains skink (*Eumeces obsoletus*), laboratory rat (*Rattus rattus*), cotton rat (*Sigmodon hispidus*), dwarf hamster (*Phodopus sungorus*), chipmunk (*Tamias striatus*), white-footed mouse (*Peromyscus leucopus*), and dog (*Canis familiaris*). All of the animals from which extracts were made were wild-caught, except the dog, laboratory rat, and dwarf hamster, which were bred in captivity. I chose species to provide chemical extracts that represent (1) species that naturally occur in the diet of the population from which the snakes were taken (white-footed mice and chipmunks); (2) species that occur in the diet of other populations of the same species (cotton rat); (3) species related to natural prey, but not sympatric with *C. horridus* (dwarf hamster and laboratory rat); (4) species sympatric with *C. horridus* that are not eaten, but that are eaten by other pitviper species (skink and green frog); and (5) a nonprey mammalian animal (dog).

The testing procedure consisted of placing a snake in a wooden, open-topped enclosure (75 × 75 × 120 cm) lined with clean construction paper and containing a water dish, a cover object, and two rectangular wooden blocks (20 × 7 × 4 cm). The snake acclimated to its new surroundings for at least 3 d before each test. At the beginning of each test, both wooden blocks were removed and paper towels were placed across the top of each. One of these paper towels, selected at random, was soaked in 10 ml of extract, while the other was soaked in tap water. Both wooden blocks were replaced in the enclosure, and the snake was allowed to respond to these chemical extracts for 120 min, after which the paper towels were removed from the wooden blocks and the trial ended. When placed back into the enclosure, the blocks were situated in an area of the arena such that each was approximately equidistant from the subject. I used this method for creating artificial chemical trails because it allows the presentation of similar chemical cues from a broad range of prey species. In pilot studies, snakes exhibited similar responses to chemical extracts made from aqueous extracts and more naturalistic trails made from allowing a small mammal to run across the substrate surface several times.

Each of the 24 subjects was tested with a different random ordering of the nine conditions (eight experimental and one control). No subject was tested more than once in a 30-d period. All tests were conducted at least 21 d after the subject had last been fed. Subjects undergoing ecdysis were not tested. Because *C. horridus* forages mainly at night (Reinert et al., 1984), all trials were conducted during the dark half of the light cycle and recorded with a video camera with low-light recording capability (Sony® Handycam CCD-TRV57).

All videotapes were coded and scored blindly. I defined the first encounter with the chemical extract as the time from the initial encounter to the time the snake removed its head from contact with the chemical extract for more than 60 sec. The snake's head did not have to be in contact with the chemical extract for the entire time of the initial encounter, so long as it was not out of contact for more than 60 sec. I recorded the latency to encounter the chemical extract and the number of tongue flicks that the snake delivered to the chemical extract during this encounter (defined as a tongue flick where the tongue actually contacted the trail or was directly above the paper towel containing the extract). Additionally, I noted whether or not the subjects adopted the stereotyped ambush posture response (Reinert et al., 1984) after encountering the chemical extract. Qualitatively, this response is a stereotyped, overt behavior, consisting of the snake coiling in a tight coil adjacent to the chemical extract, with the head and neck oriented toward the trail in a ready-to-strike position. To quantify this behavior, an ambush posture was defined as the snake not moving, with its head and anterior one third of its body in a recoiled position, oriented toward and within 15 cm of the chemical extract, adopted within 2 min of tongue flicking the chemical trail, and maintained for at least 5 min. In the majority of cases, the ambush posture was maintained for the entire duration of the trial if it was adopted.

For statistical analysis, I used a scoring system to create a composite measure of the ambush response and the number of tongue flicks, given upon first encountering a trail. This scoring system is based on a widely used measure developed for actively foraging snakes, the tongue-flick attack score, or TFAS (reviewed in Cooper and Burghardt, 1990). TFAS assumes that an attack is a stronger response than any number of tongue flicks and that the latency to attack decreases with increasing stimulus. TFAS is calculated as

$$\text{TFAS} = \text{TF}_{\text{max}} + (\text{TL} - \text{latency})$$

where TF_{max} is the maximum number of tongue flicks emitted toward the stimulus by any individual in any trial, TL is trial length in seconds, and latency is the latency in seconds to attack the stimulus. Under this scoring system, individuals that attack the stimulus receive the base unit score of TF_{max}, modified by the latency to attack. Individuals that do not attack are scored as the number of tongue flicks emitted toward the stimulus. I modified this score by substituting the ambush response for the attack response. In so doing, a tongue-flick ambush score (TFAM) is created

$$\text{TFAM} = \text{TF}_{\text{max}} + (\text{TL} - \text{latency})$$

where TF_{max} is the maximum number of tongue flicks emitted during initial encounter of the chemical extract by any individual in any trial, TL is trial length in minutes, and latency is the latency in minutes to adopt the ambush posture toward the chemical extract. This composite measure, like TFAS, assumes that an ambush posture indicates a stronger response than any number of tongue flicks, and that the latency to ambush posture decreases with increasing stimulus.

The responses of the subjects were square-root-transformed, tested for normality with Kolmogorov–Smirnov tests, and compared with a randomized block ANOVA, with snake identity as the blocking factor. Tukey's test was used to make pairwise comparisons. Statistical analysis was conducted with the software program Minitab®. All values are given as mean ± SE.

RESULTS

The number of tongue flicks toward chemical extracts during initial encounter varied markedly among treatments (Table 1) and was highest for white-footed mice (116 ± 12) and chipmunks (105 ± 13). The ambush posture was never adopted in response to the blank control treatments, nor to the skink treatment, but was adopted by at least one individual in response to all other treatments (Table 1). The ambush posture was adopted most frequently in response to chemical cues from white-footed mice (46% of trials) and chipmunks (33% of trials). In general, the mean rates of tongue flicking, latency to encounter the chemical extract, and

TABLE 1. RESPONSES OF CAPTIVE-RAISED TIMBER RATTLESNAKES DURING FIRST ENCOUNTER WITH CHEMICAL DERIVED FROM 9 DIFFERENT SOURCES

Trail	Tongue flicks	Length of encounter (min)	Latency to encounter (min)	Ambush posture occurs	TFAM
Tap water control	17 ± 5	0.6 ± 0.2	56 ± 11	0	17 ± 5
Dog	44 ± 7	1.7 ± 0.3	15 ± 7	1	52 ± 12
Green frog	42 ± 8	1.7 ± 0.3	32 ± 10	2	56 ± 15
Skink	74 ± 9	3.3 ± 0.4	18 ± 7	0	74 ± 9
Lab rat	66 ± 9	2.7 ± 0.4	27 ± 8	4	93 ± 18
Cotton rat	67 ± 11	2.8 ± 0.5	24 ± 9	5	108 ± 20
Dwarf hamster	79 ± 8	3.3 ± 0.4	11 ± 5	5	111 ± 16
Chipmunk	105 ± 13	4.0 ± 0.5	5 ± 1	8	156 ± 21
White-footed mouse	116 ± 12	5.0 ± 0.5	10 ± 4	11	164 ± 21

Note. Each snake ($N = 24$) was tested once under each treatent.

propensity to exhibit the ambush posture were correlated, indicating the usefulness of a single composite measure that takes all of these measures into account.

Comparison of the average TFAM scores with a randomized block ANOVA shows that the snakes differentiated between the treatments ($df = 8, 23$; $F = 13.2$; $P < 0.001$, Figure 1). *Post hoc* comparisons with Tukey's test reveal that (Figure 1) the response to all treatments was stronger than the response to the blank control ($P < 0.01$); the response to chemical cues from natural prey (chipmunks

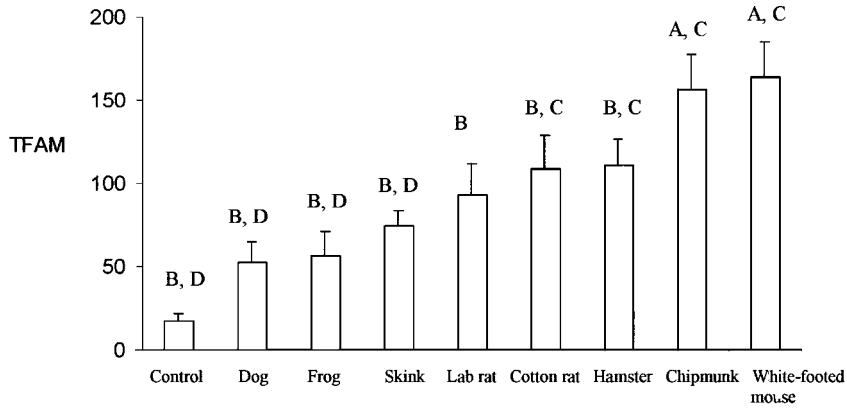

FIG. 1. Tongue flick ambush response (TFAM) of timber rattlesnakes to chemical trails from different animals ($P < 0.001$, randomized block ANOVA, $N = 24$). Tukey's test used to compare individual treatments (A > B, $P < 0.03$; C > D, $P < 0.05$).

and white-footed mice) was stronger than the response to all other treatments, including chemical cues from other small mammal species ($P < 0.03$); and the response to small mammal species was stronger than the response to trails made from nonmammal and large mammal species ($P < 0.05$).

DISCUSSION

This study illustrates the ability of *Crotalus horridus* to use chemical cues in locating suitable ambush sites. Previous studies have indicated that other ambush-foraging snake species are sensitive to chemical cues when ambushing prey. Greene (1992) reviewed field observations of venomous snakes, using chemosensory cues to locate suitable ambush sites, and coined the term "mobile ambushing" to describe that foraging strategy. Prairie rattlesnakes (*C. viridis*), in the field and in the lab, coiled near bedding soiled by potential prey (Duvall et al., 1990). Additionally, prairie rattlesnakes adopted ambush coils directed toward chemical trails left by mice (Theodoratus and Chiszar, 2000). Free-ranging pygmy rattlesnakes (*Sistrurus miliarus*) were attracted to transects that had been treated with aqueous washes from their preferred prey (Roth et al., 1999). Broad-headed snakes (*Hoplocephalus bungaroides*) were attracted to retreat sites containing odors from velvet geckos (*Oedura lesueurii*) (Downes, 1999). These studies show that the use of prey chemical cues in ambush site selection is likely an important foraging technique that is taxonomically widespread in serpents.

The use of prey kairomones (sensu Brown et al., 1970) by squamate reptiles has been the focus of several model systems in behavior and ecology (Arnold, 1981; Burghardt, 1990; Chiszar et al., 1992; Cooper, 1995); yet none of these systems have combined the ecological aspects of prey recognition and discrimination with the chemical identification of prey-derived kairomones. Identification of the kairomones used in these systems is necessary to understand the mechanisms by which predators use chemosensory information to identify and discriminate among chemical stimuli from their prey. Using bioassay-guided fractionation, proteinaceous chemoattractants that elicit predatory attacks from garter snakes in an active-foraging context have been isolated from both earthworms (Wang et al., 1993) and frogs (Wattiez et al., 1994). To date, no attempts have been made to identify kairomones used by sit-and-wait predators. Active and ambush foragers derive different types of information from prey kairomones; therefore, the nature of the kairomones used in these two contexts may differ.

Timber rattlesnakes are specialists on small mammals, and feed on almost nothing else, other than the occasional bird (Clark, 2002). The subjects from this study were taken from a north-central Pennsylvania population, where the diet consists primarily of white-footed mice, deer mice, red-backed voles, and

chipmunks (Reinert et al., 1984; Clark, 2002). The only other species used in this study that regularly occurs in the diet of timber rattlesnakes is the cotton rat, which is only eaten in southern populations (Clark, 2002). The snakes responded more positively to chemical extracts from chipmunks and white-footed mice than to those of cotton rats. Chemical extracts from lizards and frogs were not as stimulating to timber rattlesnakes as those from small mammals. Other viperid species from the same dens and populations as timber rattlesnakes prey on frogs and lizards, even though timber rattlesnakes do not (Uhler et al., 1939; Savage, 1967; Keenlyne and Beer, 1973). Taken together, these results indicate that food preferences are locally adapted, and that both intra- and interspecific variations in diet are mediated in part by the recognition of prey chemical cues.

Timber rattlesnakes responded more strongly to chemical extracts from their natural prey than to chemical extracts from closely related species. This response was exhibited in spite of the fact that the subjects were born and raised in captivity and had never encountered natural prey items. However, at least one subject exhibited the ambush posture in response to all other chemical trails, except skinks, indicating that inexperienced snakes may also investigate chemical cues from animals that do not appear in their natural diet. Therefore, even though they are biased toward natural prey items, timber rattlesnakes retain the ability to respond to novel prey. Previous studies employing the cotton applicator technique have shown that active foragers using chemical cues to trail their prey also discriminate among potential prey in favor of those that occur in the natural diet (Burghardt, 1990). As with the timber rattlesnakes in this study, actively foraging snakes also exhibit mild predatory responses to a wide range of species that do not occur in their diet. In general, it seems that snakes are born with a predisposition toward chemical cues from certain prey species, which can then be strengthened or weakened by subsequent experience (e.g., Arnold, 1978; Burghardt, 1999). Subsequent experiments with timber rattlesnakes have indicated that their responses to prey chemical cues are also affected by feeding experience (Clark, unpublished data).

The snakes in this study were raised on laboratory mice (*Mus musculus*), and thus the positive responses to chemical trails from small mammals might result from this diet. However, this does not explain why sympatric small mammal species are preferred to others. Taxonomically, chipmunks are more distantly related to *M. musculus* than any of the other small rodents used in this study (Hall, 1981), so it is unlikely that the more positive response seen to natural prey was due purely to similarity between their chemical cues and those from laboratory mice.

In addition to tongue flicking and the ambush posture, mouth gaping (Graves and Duvall, 1983) was observed in these experiments. This behavior has been observed in other species, and is presumably a mechanism by which snakes can

enhance chemosensory functioning by clearing the vomeronasal passage once it has become saturated with stimuli (Schwenk, 1995). In this study, it was most frequently seen either immediately after or immediately before an ambush posture was adopted.

Ambush site selection by timber rattlesnakes has the potential to address many questions about foraging behavior and predator–prey interactions. For example, it would be possible to examine how timber rattlesnakes respond to chemical trails that potentially encode information bearing on the probability of prey encounter or capture. Such a system could be used to test models of optimal giving-up time for an ambush forager (e.g., Nishimura, 1991) or game-theoretic model of habitat selection by prey (Bouskila, 2001).

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HOST PLANT VOLATILES SYNERGIZE RESPONSE TO SEX PHEROMONE IN CODLING MOTH, *Cydia pomonella*

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Abstract—Plant volatile compounds synergize attraction of codling moth males *Cydia pomonella* to sex pheromone (*E*,*E*)-8,10-dodecadien-1-ol (codlemone). Several apple volatiles, known to elicit a strong antennal response, were tested in a wind tunnel. Two-component blends of 1 pg/min codlemone and 100 pg/min of either racemic linalool, (E)-β-farnesene, or (*Z*)-3-hexen-1-ol attracted significantly more males to the source than codlemone alone (60, 58, 56, and 37%, respectively). In comparison, a blend of codlemone and a known pheromone synergist, dodecanol, attracted 56% of the males tested. Blends of pheromone and plant volatiles in a 1:100 ratio attracted more males than 1:1 or 1:10,000 blends. Adding two or four of the most active plant compounds to codlemone did not enhance attraction over blends of codlemone plus single-plant compounds. Of the test compounds, only farnesol was attractive by itself; at a release rate of 10,000 pg/min, 16% of the males arrived at the source. However, attraction to a 1:10,000 blend of codlemone and farnesol (42%) was not significantly different from attraction to codlemone alone (37%). In contrast, a codlemone mimic, (*E*)-10-dodecadien-1-ol, which attracted 2% males by itself, had a strong antagonistic effect when blended in a 1:10,000 ratio with codlemone.

Key Words—Sex pheromone, kairomone, synergism, host plant volatile, (*E*)-β-farnesene, (±)-linalool, (*Z*)-3-hexen-1-ol, *Malus*, *Cydia pomonella*, Tortricidae, Lepidoptera.

INTRODUCTION

Sexual communication in phytophagous insects is evidently strongly influenced by the host plant. Plant volatile compounds are known to act on the nervous and hormonal system of female moths to stimulate pheromone production and release, and to enhance attraction of male moths to female-produced sex pheromone (McNeil

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and Delisle, 1989; Raina et al., 1992; Landolt and Phillips, 1997). The interaction between sex pheromones and host plant volatiles enhances mate and host finding and therefore, also may account for reproductive isolation among closely related species. Conversely, nonhost plant volatiles have an antagonistic effect on the attraction of bark beetles to sex pheromone (Schlyter and Birgersson, 1999).

Despite a wealth of literature demonstrating the effect of plants on sex pheromone communication in the Lepidoptera, only few of the plant compounds have been identified that account for the enhancement of male moth attraction to female pheromone (Dickens et al., 1990; Meagher and Mitchell, 1998; Reddy and Guerrero, 2000).

Larvae of the codling moth *Cydia pomonella* feed on all varieties of apple, and the attraction of gravid females to apples is guided by olfactory cues (Wildbolz, 1958; Bovey, 1966; Wearing et al., 1973). The search for chemicals that mediate orientation of codling moth females to egg-laying sites has led to the discovery of a pear ester, which attracts both female and male moths (Light et al., 2001). A previous field trapping study has shown that a blend of green leaf volatiles augmented male trap captures with synthetic sex pheromone (*E*,*E*)-8,10-dodecadien-1-ol (*E*8,*E*10-12OH; codlemone) (Light et al., 1993).

Several volatile apple compounds elicit a strong antennal response in codling moth males and females (Bäckman et al., 2000; Bengtsson et al., 2001; Ansebo et al., unpublished results). Among the most active compounds are (*E*,*E*)-α-farnesene, ($\pm$)-linalool, methyl salicylate, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexenyl acetate, (*Z*)-3-hexenyl 2-methyl-butanoate, (*Z*)-3-hexenyl hexanoate, (*Z*)-3-hexenyl benzoate, butyl hexanoate, and hexyl butanoate. Two compounds, (*E*)-β-farnesene and farnesol, have in addition been shown to attract male moths in field trapping tests (Coracini et al., 2003). This wind tunnel study was done to determine whether apple volatiles serve to enhance sexual communication in codling moth.

METHODS AND MATERIALS

Insects. A laboratory strain of codling moth, reared on semiartificial diet (Mani et al., 1978) was interbred each summer with wild moths collected in apple orchards in Scania (Sweden). Newly eclosed moths were sexed daily and kept in plexiglass cages (33 $\times$ 33 $\times$ 33 cm) at 22°C under a photoperiod of L18:D6. Males used in the wind tunnel were 2-days old.

Gas Chromatography (GC). GC analysis was done on a Hewlett-Packard 6890 with a flame ionization detector on a DB-Wax column (splitless injection, 30 m $\times$ 0.25 mm ID, J & W Scientific, Folsom, CA, USA), programmed from 50°C, hold 5 min, 8°C/min to 230°C, hold 10 min, and hydrogen as carrier gas.

Chemicals. (*E*,*E*)-8,10-Dodecadien-1-ol (*E*8,*E*10-12OH; codlemone), (*E*)-10-dodecen-1-ol (*E*10-12OH), and dodecanol (12OH) were purchased from

Pherobank (Wageningen, The Netherlands) and were more than 99.5% chemically pure and >99.8% pure with respect to geometrical isomers as determined by GC. (*E*)-β-Farnesene (β-farnesene; 92.4%) and (*Z*)-jasmone (jasmone; 88.9%) were purchased from Bedoukian Research Inc. (Danbury, CT, USA), (*E*,*E*)-α-farnesene (α-farnesene; 97.3%) and ($\pm$)-linalool (linalool; 98.9%) was from Firmenich (Geneva, Switzerland), methyl salicylate (>99.9%) was from Lancaster Synthesis (Lancashire, UK), and farnesol (90.7%) and (*Z*)-3-hexen-1-ol (*Z*3-6OH; >99.9%) were obtained from Sigma-Aldrich GmbH (Sternheim, Germany). (*Z*)-3-Hexenyl acetate (*Z*3-6Ac; >99.9%), (*Z*)-3-hexenyl 2-methyl-butanoate (>99.9%), (*Z*)-3-hexenyl hexanoate (98.2%), (*Z*)-3-hexenyl benzoate (99.0%), butyl hexanoate (>99.9%), and hexyl butanoate (95.1%) were synthesized from the corresponding acid and alcohol precursors as described elsewhere (Coracini et al., 2003).

Wind Tunnel. Wind tunnel hardware, odor application system, flight tracker, and test protocol were described by Witzgall et al. (2001). The wind tunnel has a flight section of 63 × 90 × 200 cm and was lit diffusely from above at 6 lux. Wind speed was 30 cm/sec, and the room temperature was 22°C.

Dilutions of synthetic compounds in redistilled ethanol (Solveco Chemicals AB, Malmö, Sweden) were released from an ultrasound evaporator (Gödde et al., 1999). A motor-driven syringe delivers pheromone solutions at a rate of 10 μl/min through Teflon tubing to a capillary tube with a drawn-out tip. A piezo-ceramic disc vibrates the glass capillary at ca. 100 kHz, producing an aerosol, which evaporates a few centimeters downwind from the tip of the capillary. This "sprayer" allows application of chemicals at a constant rate and known chemical purity.

Three batches of fifteen 2-day-old males were tested per day, within 1 to 3 hr after the onset of scotophase. Males were transferred from their holding cage to glass tubes (2.5 × 12.5 cm) stoppered with gauze, ca. 15 min before testing. Each blend was tested four times ($N = 60$ males) on different days. The upwind end of glass tubes holding the males was ca. 180-cm downwind from the source. The following types of behavior were recorded: activation (walking and wing-fanning; A), taking flight (F), flying upwind over 50, 100, and 150 cm toward the source (50; 100; 150), touching the source (S), and landing at the source, followed by walking while wing-fanning (L).

Statistical Analysis. The numbers of males recorded in the wind tunnel were transformed to $\log(x + 1)$ and statistically evaluated by one-way ANOVA followed by Duncan's multiple range test. Significance level was set at 0.05.

RESULTS

Male codling moths were flown to two-component blends of the main pheromone component codlemone (*E*8,*E*10-12OH), 12 apple volatiles known

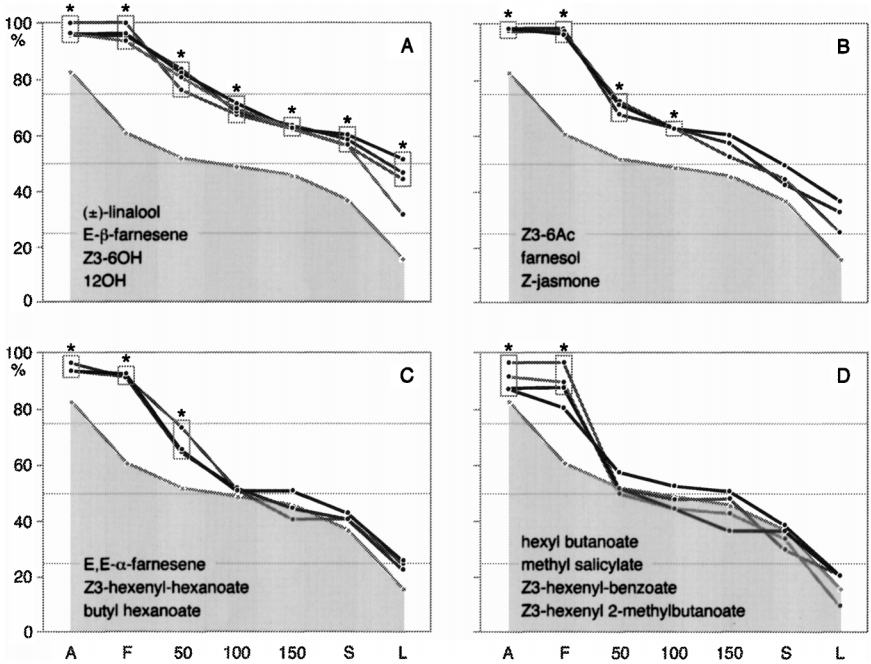

FIG. 1. Wind tunnel response of codling moth males *C. pomonella* ($N = 60$) to two-component blends of main pheromone compound codlemone $E8,E10$-12OH (1 pg/min) and apple volatiles (100 pg/min). Activation from rest (A), taking flight (F), flying upwind over 50, 100, and 150 cm toward the source, source contact (S), landing and wing-fanning at source (L). Asterisks show significant differences between codlemone alone (shaded curve) and two-component blends (ANOVA followed by Duncan's test; $P < 0.05$). Compounds are ranked according to the number of males landing at the source.

to elicit a strong antennal response (Ansebo et al., unpublished results), a floral compound, jasmone, and a pheromone synergist, 12OH (Arn et al., 1985).

Codlemone, released at 1 pg/min, was blended with these compounds in a 1:100-ratio (Figure 1). Blends of codlemone and linalool, β-farnesene or $Z3$-6OH significantly enhanced the entire male behavioral sequence, from activation to landing. In response to these blends, 44 to 51% of the males landed and wing-fanned on the source, significantly more than with codlemone alone at the same release rate (16%; Figure 1A) or codlemone at a 10-fold release rate (23%; data not shown). Fewer males landed and wing-fanned at a blend source of codlemone and 12OH, a known female-produced pheromone synergist (Figure 1A).

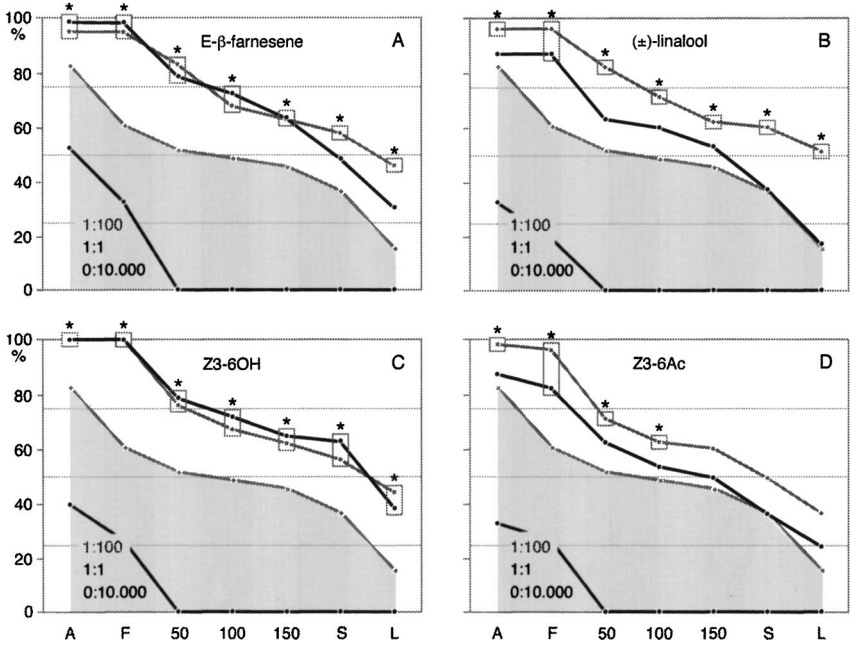

FIG. 2. Wind tunnel response of codling moth males *C. pomonella* ($N = 60$) to apple volatiles (1 and 100 pg/min), with and without the main pheromone compound codlemone *E*8,*E*10-12OH (1 pg/min). Asterisks show significant differences between codlemone alone (shaded curve) and two-component blends (ANOVA followed by Duncan's test; $P < 0.05$). Legend as in Figure 1.

Jasmone, farnesol, and *Z*3-6Ac had an intermediate effect on male attraction to codlemone. Upwind flights up to 100 cm were enhanced, but the number of males reaching the source was not significantly different from codlemone alone (Figure 1B). Butyl hexanoate, (*Z*)-3-hexenyl hexanoate, and α-farnesene stimulated more males to fly upwind, over 50 cm towards the source, but attraction over 100 cm or more was not different from codlemone (Figure 1C). The remaining four compounds had an effect only on male activation, but not on the male upwind flight response (Figure 1D).

Several compounds were tested at blend ratios of 1:1 and 1:10,000 pg/min, but none of these blends attracted more males than the respective 1:100-blends. Representative results are shown in Figures 2 and 3. The 1:100 blend ratio was then tested at three doses of codlemone (1, 10, and 100 pg/min), two of which (1:100, 100:10,000) are shown in Figure 3. For the 100:10,000-blends, only the number of males taking flight was different from codlemone alone.

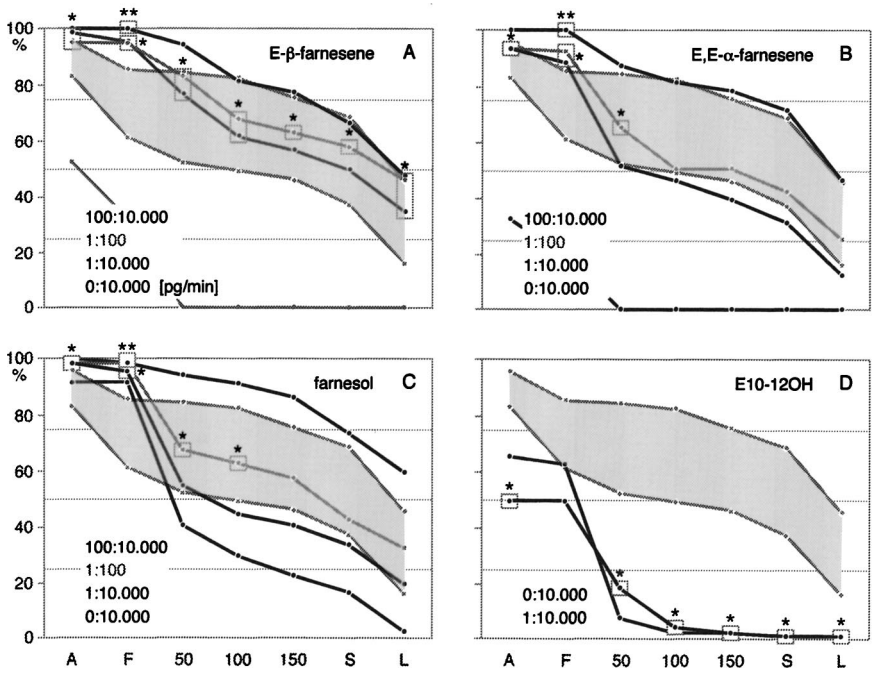

FIG. 3. Wind tunnel response of codling moth males *C. pomonella* ($N = 60$) to three apple volatiles and *E*10-12OH (1, 100, or 10,000 pg/min), with and without the main pheromone compound codlemone *E*8,*E*10-12OH (1 or 100 pg/min). Significant differences between two-component blends and codlemone at two different doses, 1 pg/min and 100 pg/min, are marked by one and two asterisks, respectively (ANOVA followed by Duncan's test; $P = 0.05$). Legend as in Figure 1.

β-Farnesene, linalool, and *Z*3-6OH alone did not attract males (Figure 2). Farnesol, on the other hand, did not enhance attraction to codlemone, but attracted males as a single compound (Figure 3C). In comparison, the monoene *E*10-12OH was a weak attractant, 2% of the test males touched the source. When *E*10-12OH was blended with codlemone (1:10,000 pg/min), male attraction was almost abolished (Figure 3D).

Codlemone (1 pg/min) was blended with two (farnesene, linalool) or four (β-farnesene, linalool, *Z*3-6Ac, and *Z*3-6OH) of the most active compounds. Compounds were added at either equal or 100-fold amounts (1:1:1; 1:100:100; 1:1:1:1:1; 1:100:100:100:100). None of these three- and five-component blends was significantly more attractive (data not shown) than the two-component blend of codlemone and linalool (Figure 1A).

DISCUSSION

The integration of host plant chemistry and insect-produced signals is a matter of concern in the study of mate finding and reproductive isolation among related species. Host plant volatiles influence sex pheromone communication probably in most phytophagous insects by enhancing both pheromone production and response (McNeil and Delisle, 1989; Landolt and Phillips, 1997). However, there are few studies on the chemicals responsible for host plant enhancement of pheromonal communication in Lepidoptera, probably because male moths become readily attracted to female or synthetic sex pheromone even in the absence of host volatiles (Dickens et al., 1990; Light et al., 1993; Meagher and Mitchell, 1998; Reddy and Guerrero, 2000).

We show that attraction of codling moth *C. pomonella* to the main pheromone compound codlemone is greatly enhanced by addition of either (*E*)-β-farnesene, ($\pm$)-linalool, or *Z*3-6OH (Figures 1 and 2). A similar effect is obtained with pheromone synergists, such as 12OH (Figure 1A; Arn et al., 1985) or small amounts of E8,Z10-12OH and E8,E10-12Ac (Witzgall et al., 2001). All plant volatiles tested, except (Z)-jasmone, have been identified from green apple leaves and fruits (Bengtsson et al., 2001).

A field study by Light et al. (1993) showed increased trap captures of *C. pomonella* males in response to blends of codlemone plus a five-component blend of green-leaf volatiles, in which *Z*3-6OH and *Z*3-6Ac were the most abundant components. Both *Z*3-6OH and *Z*3-6Ac had a significant effect in our wind tunnel experiments (Figures 1 and 2); linalool and β-farnesene are attractants for several other insects (Landolt et al., 1994; Al-Abassi et al., 2000; Koschier et al., 2000; Bruce and Cork, 2001; Borg-Karlson et al., 2003).

The synergistic effect of β-farnesene was strongest at the lowest codlemone dose tested (1 pg/min), as this is the case with pheromone synergists in codling moth (Arn et al., 1985; Witzgall et al., 2001). In contrast to pheromone synergists, blend ratio was not as critical when adding plant volatiles to pheromone. Amounts of 1 to 10,000 pg/min β-farnesene added to 1 pg/min codlemone augmented male attraction (Figures 2 and 3). Codling moth males are tuned to a narrow range of pheromone blends, probably because the female pheromone signal is quite constant with respect to release rate and blend proportion (Bäckman et al., 1997). Apple volatile compounds, in comparison, are present in large amounts in orchard air (Bäckman, 1997), but substantial fluctuations are expected to occur over time and space, especially in mixed vegetation.

Tests with 1:1 blends (Figure 2) demonstrate that the male response to plant compounds is both specific and sensitive. This is supported by single sensillum recordings in codling moth and other Lepidoptera, showing that plant volatiles are perceived by specialized chemoreceptor neurons (Masson and Mustaparta, 1990; Anderson et al., 1995; Bäckman et al., 2000; Røstelien et al., 2000).

In *Helicoverpa zea*, a male receptor neuron has recently been discovered, which responds to a blend of the main pheromone compound (*Z*)-11-hexadecenal with either linalool or *Z*3-6OH, but not to the plant compounds alone (Ochieng et al., 2002). This might explain why linalool, β-farnesene, and *Z*3-6OH have a clear synergistic effect in codling moth when blended with pheromone, but have no behavioral effect as single compounds (Figure 2). Farnesol, on the other hand, is more likely perceived by a separate sensory channel, as it attracted a significant number of males by itself (Figure 3C). Farnesol is not a codlemone mimic; addition of 10 ng/min farnesol to 1 pg/min codlemone had an additive effect on the male response. The monoene *E*10-12OH, in comparison, presumably acts on the codlemone receptor neuron—it attracted few males at a release rate of 10 ng/min, but blocked male attraction almost completely when blended with codlemone (Figure 3D; Preiss and Priesner, 1988).

A synergistic effect between pheromone and plant volatile compounds may evidently subserve mate finding. Some apple volatiles, such as β-farnesene, will, according to our study, increase communication distances by amplifying weak pheromone signals (Figure 3A).

Furthermore, males were attracted to farnesol, a compound that has been identified from apple (Figure 3C, Matich et al., 1996). Males will greatly improve reproductive success by locating host plants, i.e., sexual rendezvous sites, even before females start to release pheromone. Field studies confirm that both codling moth and pea moth males, *C. nigricana*, become attracted to the host plant well before the onset of female calling (Witzgall et al., 1996, 1999). Mating success should be highest for those males that quickly arrive at the female, from a short distance.

Synergism between host plant volatiles and sex pheromone also is likely to play an important role in reproductive isolation. An antagonistic effect of nonhost volatiles on attraction to pheromones provides further evidence for this concept (Schlyter and Birgersson, 1999; McNair et al., 2000; Bedard et al., 2002). Lepidopteran pheromones are thought to be rather species-specific. Nevertheless there are many examples of similar pheromone blends, especially among species occurring on different host plants (Arn et al., 2000). For example, the green budworm moth *Spilonota ocellana* and its sibling species, the red larch budworm *S. laricana* seemingly produce the same pheromone blend, and males court the respective heterospecific calling females in the laboratory. However, traps baited with a two-component pheromone attract *S. laricana* in larch trees, while the same traps attract *S. ocellana* when placed in apple trees (Witzgall et al., 1991).

A central question remaining to be answered concerns the specificity of plant signals. The compounds investigated in this study are found in host plants, as well as many nonhost plants of codling moth. Blends of single-plant compounds and sex pheromone are specific. In contrast, host signals attracting either females or males outside the female calling period must naturally be multi-compound to convey

specificity. Multi-compound blends of several plant volatiles and pheromone did not increase male attraction, as compared to blends of single-plant volatiles and pheromone in this study. Multi-compound blends will instead be tested for male attraction without pheromone, and for attraction of female moths.

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SYNTHESIS AND BIOLOGICAL ACTIVITY OF THE FOUR STEREOISOMERS OF 4-METHYL-3-HEPTANOL: MAIN COMPONENT OF THE AGGREGATION PHEROMONE OF *Scolytus amygdali*

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Abstract—Stereoisomers of 4-methyl-3-heptanol are major components of aggregation pheromones of bark beetles and trail pheromones of ants. Recently, (3*S*,4*S*)-4-methyl-3-heptanol (**I**) has been tentatively identified as the main component of the aggregation pheromone of the almond bark beetle, *Scolytus amygdali* (Coleoptera: Scolytidae). The four stereoisomers of 4-methyl-3-heptanol were prepared and bioassayed. Key steps included preparation of chiral 4-methyl-3-heptanones using SAMP and RAMP reagents, reduction to the corresponding alcohols, and stereospecific transesterification with vinyl acetate with lipase AK catalysis. In field tests, only (3*S*,4*S*)-4-methyl-3-heptanol attracted beetles in combination with the synergist (3*S*,4*S*)-4-methyl-3-hexanol, whereas (3*R*,4*S*)- and (3*R*,4*R*)-4-methyl-3-heptanols were inhibitory.

Key Words—4-Methyl-3-heptanol, stereoisomers, chirality, lipase, biological activity, *Scolytus amygdali*, bark beetle, Scolytidae, Coleoptera, aggregation pheromone.

INTRODUCTION

Stereoisomers of 4-methyl-3-heptanol are components of several aggregation and trail pheromones of insects. The (3*S*,4*S*)-isomer (**I**) is the major component of the aggregation pheromone of the bark beetles *Scolytus scolytus* and *S. multistriatus* (Pearce et al., 1975; Blight et al., 1979a,b; Pignatello and Grant, 1983).

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The (3*R*,4*S*)-isomer (**II**) is the trail pheromone of the ant *Leptogenys diminuta* (Attygalle et al., 1988; Steghaus-Kovac et al., 1992). Other stereoisomers were found in the leaf-cutter ant *Atta laevigata* (Hernandez et al., 1999). Recently, (3*S*,4*S*)-4-methyl-3-heptanol (**I**) was tentatively identified as the main component of the aggregation pheromone of the almond bark beetle, *Scolytus amygdali* Geurin-Meneville (Coleoptera: Scolytidae) along with (3*S*,4*S*)-4-methyl-3-hexanol, which functions as a synergist. Substitution of the *S*,*S*-isomer (**I**) with racemic 4-methyl-3-heptanol resulted in a lower trap catch of beetles in the field, indicating the possibility that one or more of the other stereoisomers was inhibitory (Ben-Yehuda et al., 2002).

The objective of this study was to develop syntheses of all four stereoisomers of 4-methyl-3-heptanol, and to assess their biological activity towards the almond bark beetle. A number of syntheses have been described in the literature, but some are complex, whereas others provide compounds with low enantiomeric purity. Here we present simple routes on the basis of enzyme-catalyzed transesterification of two diastereoisomeric mixtures of two 4-methyl-3-heptanol isomers that provide all four isomers in high chemical and enantiomeric purity. We also report the response of *S. amygdali* to the purified stereoisomers.

METHODS AND MATERIALS

Chemicals. Commercial chemicals were used without further purification unless otherwise stated. 4-Methyl-3-heptanol, 3-pentanone, (*S*)-(−)-1-amino-2-(methoxymethyl) pyrrolidine (SAMP), (*R*)-(+)-1-amino-2-(methoxymethyl) pyrrolidine (RAMP), diisopropylamine, BuLi (1.6 M in hexane), propyl iodide, $LiAlH_4$, vinyl acetate, molecular sieves (4 Å), and *t*-butylmethyl ether were purchased from Aldrich (Milwaukee, WI). Silica gel 60 and KOH were purchased from Merck (Darmstadt, Germany). Lipase AK, *Pseudomonas fluorescens*, was a gift from Amano-Enzyme (Nagoya, Japan). Enantiomerically pure (3*S*,4*S*)-4-methyl-3-heptanol and (3*S*,4*S*)-4-methyl-3-hexanol were obtained from W. Francke, Hamburg University. Ether was distilled over sodium-benzophenone, CH_2Cl_2 over CaH_2, and propyl iodide over K_2CO_3.

Analytical Procedures. Reactions were monitored on an Hewlett-Packard 6890 GC (Palo Alto, California) equipped with a flame ionization detector and a split/splitless injector. Helium was used as the carrier gas with a constant flow rate of 1.5 ml/min for all capillary columns. An HP5 column (30 m × 0.25 mm ID, 0.25-μm film thickness) was used routinely. The initial column temperature was 70°C for 2 min, then 20°/min to 180°C for the analysis of the hydrazones, and 60°C for 8 min, then 20°/min to 140°C for the analysis of ketones and alcohols. The injector and detector were maintained at 220°C and analyses were conducted in the splitless mode. The enantiomers of 4-methyl-3-heptanone were analyzed on a chiral

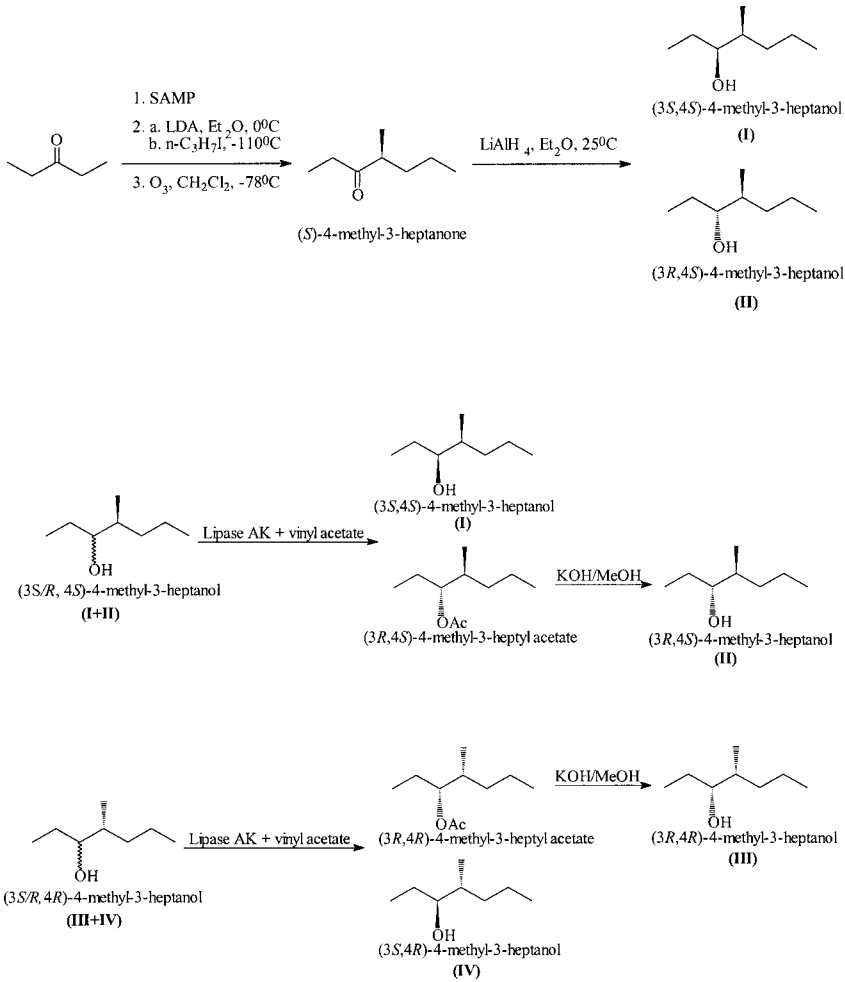
Rt-βDEXsm (Restek, Bellefonte, Pennsylvania) column (30 m $\times$ 0.25 mm $\times$ 0.25 μm film thickness) isothermally at 70°C. The analyses of stereoisomers of 4-methyl-3-heptanol and the corresponding acetates were performed on a chiral Lipodex G, octakis-(2,3-di-*O*-pentyl-6-*O*-methyl)-γ-cyclodextrin column (25 m $\times$ 0.25 mm $\times$ 0.25 μm film thickness) (Macherey Nagel, Duren, Germany) that was held at 55°C for 10 min, then programmed to 100°C at 2°C/min. Ozonolysis was performed with a Fisher (Meckenheim/Bonn, Germany) ozone generator, model 505. Optical rotations of the 4-methyl-3-heptanol stereoisomers were measured on a Perkin-Elmer polarimeter, model 341, in a cell of 1-cm cavity length at 589 nm. Electron impact ionization (EI) mass spectra were obtained on an Agilent 6890N GC-MS (Little Falls, DE, USA) instrument equipped with an HP5MS column (30 m $\times$ 0.25 mm ID, 0.25-μm film thickness) at 70 eV. The oven temperature was programmed as described above for the HP5 column. The mass spectra were compared with those in the Wiley 7 and NIST libraries. Proton NMR spectra were recorded on a Brucker 300 MHz machine in $CDCl_3$ with TMS as internal standard.

Synthesis of the Stereoisomers of 4-Methyl-3-heptanol. The synthesis is outlined in Scheme 1.

(S)-4-Methyl-3-heptanone and (R)-4-Methyl-3-heptanone. The synthesis of the chiral ketones was conducted according to the procedure of Enders et al. (1993), using SAMP (*S*-enantiomer) and RAMP (*R*-enantiomer), respectively. The ketones were purified by column chromatography on silica gel with pentane +1% ether as eluent, rather than by distillation, to avoid racemization. The proton NMR spectra of the ketones were identical with those reported by Enders et al. (1993). The optical purities of the ketones were 97–98% as determined by GC analysis on a chiral column.

(3S/R,4S)-4-Methyl-3-heptanol. $LiAlH_4$ (0.7 g) was added to (*S*)-4-methyl-3-heptanone (718 mg) in 20 ml dry ether under argon. The reaction was monitored by GC and terminated after 4 hr. The mixture was poured on ice and extracted twice with 10 ml of ether. Most of the ether was removed by distillation and the last traces were evaporated with a gentle flow of nitrogen to yield 660 mg of a 1:1 mixture of the two diastereoisomeric alcohols (**I** + **II**).

*Enzymatic Separation of the (3S/R,4S)-4-Methyl-3-heptanols (***I** + **II***).* The mixture of the two alcohols (660 mg) was dissolved in 15 ml *t*-butylmethyl ether in a 50 ml flask equipped with a stopcock and a magnetic stirrer. A few oven-dried molecular sieve pellets and lipase AK (60 mg) were added. The heterogeneous mixture was stirred at room temperature and 1 ml vinyl acetate was added to start the transesterification. The reaction was monitored by periodic GC analysis on a chiral column. When the conversion had reached ~50%, the mixture was filtered and the solvent was evaporated with a flow of nitrogen. The residue, containing acetate and alcohol, was separated by column chromatography on silica gel (10 g), with pentane and then with pentane +1% ether as eluent. The fractions were monitored by GC. The acetate eluted first, yielding 255 mg of (3*R*,4*S*)-4-methyl-3-heptyl



SCHEME 1. Synthesis of the four stereoisomers of 4-methyl-3-heptanol.

acetate, followed by 227 mg of (3*S*,4*S*)-4-methyl-3-heptanol (**I**). Optical rotation of the alcohol $[\alpha]_D^{25} - 19.96^{\circ}$ ($c = 1.59$, hexane) ($[\alpha]_D^{25} - 22.02^{\circ}$, $c = 1.1$, hexane; Tripathy and Matteson, 1990); NMR, δ 3.42 (m, 1H, CHO), 1.12–1.57 (m, 8H, $3CH_2$+CH+ OH), 0.85–0.97 (m, 9H, $3CH_3$); MS, m/z (%) 112 (2, $M^+ - H_2O$), 101 (17, $M^+ - C_2H_5$), 83 (21, $M^+ - C_2H_7O$), 59 (100, $M^+ - C_5H_{11}$).

(3R,4S)-4-Methyl-3-heptanol. (3*R*,4*S*)-4-Methyl-3-heptyl acetate (**II-Ac**) (255 mg) was hydrolyzed with 3 ml methanolic KOH (2.5 M). After 6 hr, 2 g of ice was added to the stirred reaction mixture and the alcohol was extracted

three times with 5 ml pentane. The organic phase was washed with 3 ml HCL (1 M), followed by 3 ml of $NaHCO_3$ (sat) and 3 ml NaCl (sat), and subsequently dried over $MgSO_4$. Evaporation of the solvent gave 150 mg (3*R*, 4*S*)-4-methyl-3-heptanol. Rotation $[\alpha]_D^{25} - 10.23^\circ (c = 1.733$, hexane) ($[\alpha]_D^{25} - 11.98^\circ$, $c = 0.6$, hexane; Tripathy and Matteson, 1990); NMR, δ 3.35 (m, 1H, CHO), 1.05–1.6 (m, 8H, $3CH_2$+CH+OH), 0.87–0.99 (m, 9H, $3CH_3$); MS, 112 (1, $M^+ - H_2O$), 101 (26, $M^+ - C_2H_5$), 83 (37, $M^+ - C_2H_7O$), 59 (100, $M^+ - C_5H_{11}$).

*(3S/R,4R)-4-Methyl-3-heptanols (***III** + **IV***).* These two diastereoisomeric alcohols were prepared following the same procedure as above, starting with (*R*)-4-methyl-3-heptanone. The two alcohols were separated by enzymatic transesterification. The (3*R*, 4*R*) stereoisomer was esterified preferentially. The yields of the (3*S*/*R*, 4*R*)-4-methyl-3-heptanols (**III** + **IV**) were similar to those of the (3*S*/*R*, 4*S*)-4-methyl-3-heptanols (**I** + **II**). They were characterized by their retention times on the HP5 column and by their MS spectra which were identical to those of the corresponding enantiomers. The stereoisomeric purity of all four 4-methyl-3-heptanols ranged from 89 to 95% (Table 1).

Acetylation. Racemic and chiral 4-methyl-3-heptanols were acetylated with acetic anhydride and pyridine at room temperature (milligram scale) for GC analysis to determine their chemical and enanatiomeric purity. Reaction mixtures were diluted with cold water and extracted with hexane. The organic phase was washed with dilute HCl, saturated aqueous $NaHCO_3$, and dried over anhydrous $MgSO_4$.

Field Experiments. Field experiments were conducted at Kefar Tavor in eastern Galilee in small blocks of almond, plum, apricot, and nectarine. Treatments were arranged in randomized plots with eight replicates of each. Black flat funnel traps (Röchling, Haren, KG) were suspended 1 m above the ground between pairs of trees at intervals of 30–50 m. Polyethylene dispensers (Just Plastic, Norwich, UK) were impregnated with either (1) 200 μg of each stereoisomer of 4-methyl-3-heptanol combined with 100 μg of the synergist, (3*S*,4*S*)-4-methyl-3-hexanol, or (2) a binary mixture of (3*S*,4*S*)-4-methyl-3-heptanol + (3*S*,4*S*)-4-methyl-3-hexanol and one of the other three stereoisomers of 4-methyl-3-heptanol. All

TABLE 1. CHEMICAL AND CHIRAL PURITY OF THE FOUR STEREOISOMERIC 4-METHYL-3-HEPTANOLS

Isomer\Impurity	3*S*,4*S*	3*R*,4*S*	3*R*,4*R*	3*S*,4*R*	de^a(%)
3*S*,4*S* (**I**)	**94.55**	2.94	0.83	1.19	94.0
3*R*,4*S* (**II**)	2.35	**95.08**	0.81	0	95.2
3*R*,4*R* (**III**)	0.59	3.76	**92.4**	1.61	96.6
3*S*,4*R* (**IV**)	2.38	0	8.03	**89.3**	83.5

[a] Diastereoisomeric excess, calculated by $(|A - B|)/(|A + B|) \times 100$; where A and B are the relevant diastereoisomers which were separated. Each pair of diastereoisomers that was separated contained a few percent of the other diastereoisomeric pair originating from the enantiomeric 4-methyl-3-heptanone.

blends were applied in hexane, and pure hexane was used as a control. The pheromone traps were deployed for four 7-d periods. Traps were emptied every week, and their locations were rotated by one position. Trap catches were transformed to $(x + 0.5)^{0.5}$ and subjected to ANOVA (analysis of variance), followed by a Student–Neuman–Keuls test at $P < 0.05$.

RESULTS

Synthesis of the Four Stereoisomers of 4-Methyl-3-heptanol. The key step in syntheses of the four stereoisomers of 4-methyl-3-heptanol was the stereoselective enzymatic transesterification of the two diastereoisomeric pairs of (3*S*,4*S*)-4-methyl-3-heptanol (**I**) +(3*R*,4*S*)-4-methyl-3-heptanol (**II**) and (3*R*,4*R*)-4-methyl-3-heptanol (**III**) +(3*S*,4*R*)-4-methyl-3-heptanol (**IV**). Both pairs were prepared by the $LiAlH_4$ reduction of (*S*)-4-methyl-3-heptanone and (*R*)-4-methyl-3-heptanone that were prepared according to Enders et al. (1993).

The best enzymatic separation of each of the two diastereoisomeric pairs was achieved with Lipase AK in *t*-butylmethyl ether. All four stereoisomers could be resolved on the LipodexG capillary column (König, 1992); however, while monitoring the enzymatic transesterification, we observed that the corresponding acetates separated better than the alcohols. The final chemical and chiral purities of all four stereoisomeric 4-methy-3-heptanols were, therefore determined with the corresponding acetates (Figure 1, Table 1).

Capture of Scolytus amygdali in Traps Baited with the Stereoisomers of 4-Methyl-3-heptanol. The attractiveness of each of the four stereoisomers of 4-methyl-3-heptanol, in combination with (3*S*,4*S*)-4-methyl-3-hexanol acting as a synergist, was compared in the first field test. Both male and female *S. amygdali*

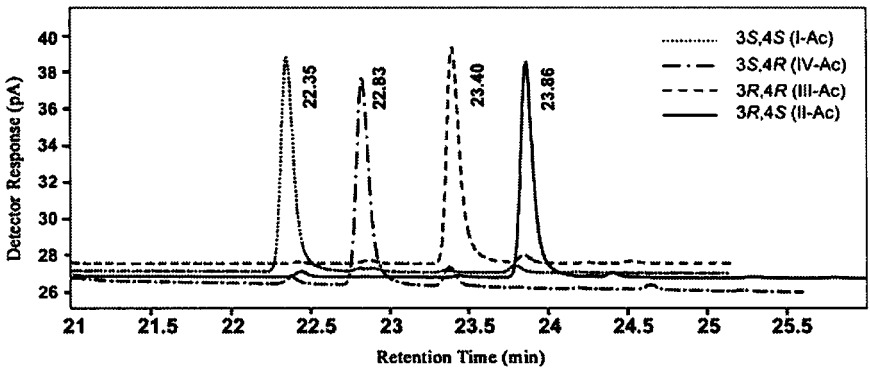


FIG. 1. GC analysis of the acetates of the four 4-methyl-3-heptanols on the Lipodex-G γ-cyclodextrin column (55°C for 10 min then 2°C/min to 100°C).

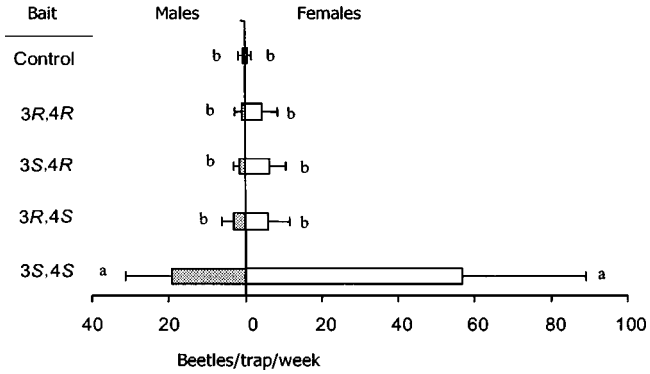


FIG. 2. Mean number of *S. amygdali* captured in traps ($N = 8$) baited with the four stereoisomers of 4-methyl-3-heptanol and the synergist, (3*S*,4*S*)-4-methyl-3-hexanol. Traps were exposed in a mixed stone fruit orchard in May 2002 for 4 wk. Means followed by the same letter (total adults, males and females) are not significantly different at $P < 0.05$.

are attracted to (3*S*,4*S*)-4-methyl-3-heptanol (**I**) when tested with the synergist (Figure 2). The small numbers of beetles trapped in response to the other stereoisomers were no different from the hexane control (Table 1).

The second field test assessed whether one or more of the unattractive stereoisomers were inhibitory. The attractiveness of a combination of (3*S*,4*S*)-4-methyl-3-heptanol (**I**) with the synergist was compared with those of mixtures containing this blend and each of the other stereoisomers. The results indicated that (3*R*,4*R*)-4-methyl-3-heptanol (**III**) and (3*R*,4*S*)-4-methyl-3-heptanol (**II**) reduced the number of males trapped whereas (3*S*,4*R*)-4-methyl-3-heptanol (**IV**) had no effect on males. Trap catches of females were not affected by **II**, **III**, or Figure 3.

DISCUSSION

A number of syntheses of the stereoisomers of 4-methyl-3-heptanol have been published. Some address the preparation of only one stereoisomer (Frater, 1979; Dominguez et al., 1991; Mori, 1992; Agami et al., 1996; Li et al., 1999a,b) whereas others deal with the synthesis of all four (Tripathy and Matteson, 1990; Unelius et al., 1998). Most are multistep syntheses or provide stereoisomers of low chiral purity. Recently, we elucidated the almond bark beetle aggregation pheromone, of which (3*S*,4*S*)-4-methyl-3-heptanol was tentatively identified as the major component and (3*S*,4*S*)-4-methyl-3-hexanol was found to be a minor synergistic component (Ben-Yehuda et al., 2002). The four stereoisomers of

4-methyl-3-heptanol were required to confirm the identification of (3*S*,4*S*)-4-methyl-3-heptanol as the active component and to establish whether any of the other stereoisomers were inhibitory.

We decided to assess the possibility of using the lipase-catalyzed transesterification for the separation of diastereoisomeric rather than enantiomeric pairs. Although the use of lipase enzymes for resolution of racemic alcohols is common, it has been applied to diastereoisomeric mixtures in only a small number of cases (Morgan et al., 1999). Also, attempted separation of racemic erythro- and threo-4-methyl-3-heptanols (separation of enantiomers) by means of a lipase (*Candida antarctica*) catalyzed transesterification resulted in low enantioselectivity (Unelius et al., 1998).

The feasibility of separation of the diastereoisomeric mixtures was tested with a mixture of all four stereoisomers of 4-methyl-3-heptanol. We evaluated three solvents-hexane, THF, and *t*-butylmethyl ether, and two enzymes-lipase PS (*Pseudomonas cepacia*) and lipase AK (*Pseudomonas fluorescens*). The best results were obtained with lipase AK in *t*-butylmethyl ether. The resulting rates of transesterification of the four alcohols with vinyl acetate diminished in the order (3*R*,4*S*) (**II**) > (3*R*,4*R*) (**III**) > (3*S*,4*S*) (**I**) > (3*S*,4*R*) (**IV**). The percentages of the stereoisomeric alcohols that remained after transesterification for 8 days were **I** (94%), **II** (0%), **III** (27%), and **IV** (100%). The results indicated that each of the two diastereoisomeric pairs of (3*S*,4*S*)-4-methyl-3-heptanol (**I**) + (3*R*,4*S*)-4-methyl-3-heptanol (**II**) and (3*R*,4*R*)-4-methyl-3-heptanol (**III**) + (3*S*,4*R*)-4-methyl-3-heptanol (**IV**) could be separated by enzymatic transesterification. The separation between the (3*R*,4*S*) and (3*S*,4*S*) diastereoisomers was complete after 1 wk, when the (3*R*,4*S*) alcohol was completely converted into the corresponding acetate. The reaction must be monitored carefully to terminate it before significant amounts of the (3*S*,4*S*) alcohol start to react. The separation between the (3*R*,4*R*) and (3*S*,4*R*) diastereoisomers was slower, taking 3 wk and requiring the addition of a second portion of enzyme. Furthermore, (3*R*,4*R*)-4-methyl-3-heptanol was not completely acetylated, even after the addition of more enzyme.

To our knowledge, this is the first enzyme-catalyzed separation of diastereoisomeric pairs of methyl alkanols with two adjacent chiral centers. The small percentages of the other stereoisomers in the final samples probably originated from the commercial SAMP and RAMP reagents that were 95–97% pure, providing 4S-methyl-3-heptanone and 4*R*-methyl-3- heptanone with 95–97% enantiomeric purity. The new route facilitated the preparation of all four stereoisomers of 4-methyl-3-heptanol with high chemical and chiral purity and enabled the evaluation of their biological activity for the almond bark beetle.

The field results presented in Figure 2 confirm the identification of **I** as the main component of the almond bark beetle aggregation pheromone (Ben-Yehuda et al., 2002). The second field bioassay indicated that (3*R*,4*S*)-4-methyl-3-heptanol (**II**) and (3*R*,4*R*)-4-methyl-3-heptanol (**III**) were inhibitory to males

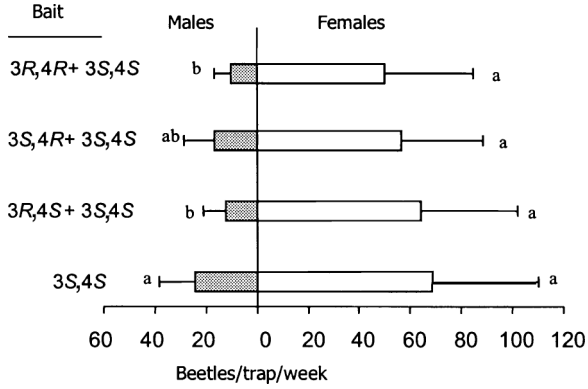


FIG. 3. Mean number of *S. amygdali* captured in traps baited with (3*S*,4*S*)-4-methyl-3-heptanol and the synergist, (3*S*,4*S*)-4-methyl-3-hexanol and mixtures containing this blend with each of the three other stereoisomers of 4-methyl-3-heptanol. Traps ($N = 8$) were exposed in a mixed stone fruit orchard in June 2002 for 4 wk. Means followed by the same letter (separately for total adults, males and females) are not significantly different at $P < 0.05$.

but not females (Figure 3). This finding clarified the previous observation that the racemic mixture of all four 4-methyl-3-heptanol steroisomers was less attractive than (3*S*,4*S*)-4-methyl-3-heptanol (**I**) (Ben-Yehuda et al., 2002).

Conversely, the unnatural stereoisomers of (3*S*,4*S*)-4-methyl-3-heptanol (**I**), which is also the main aggregation pheromone component of *S. scolytus* and *S. multistriatus*, did not affect the trap catch of these beetles (Blight et al., 1979b). Similarly, the attractiveness of *Rhynchophorus* palm weevils to their aggregation pheromones, which are also (*S*,*S*)-methyl alkanols with two adjacent chiral centers, was not affected by their unnatural stereoisomers (Perez et al., 1994; Giblin-Davis et al., 1996, and literature cited therein).

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IDENTIFICATION OF THE SEX PHEROMONE OF THE CURRANT SHOOT BORER *Lampronia capitella*

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Abstract—Under an artificial light:dark cycle, females of *Lampronia capitella* were observed calling, with extended terminal abdominal segments, during the first 2 hr of the photoperiod. Extracts of terminal abdominal segments from females elicited large electroantennographic responses from male antennae. Gas chromatography with electroantennographic detection revealed three active peaks. Based on comparison of retention times and mass spectra of synthetic standards, these compounds were identified as (Z,Z)-9,11-tetradecadienol and the corresponding acetate and aldehyde. The electroantennographic activity of the four geometric isomers of all three compounds was investigated, and the respective (Z,Z)-isomer was found to be the most active in all cases. Aldehydes generally elicited larger antennal responses than alcohols, whereas acetates were the least active compounds. A subtractive trapping assay in the field,

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based on a 13:26:100 μg mixture of (Z,Z)-9,11-tetradecadienal, (Z,Z)-9,11-tetradecadienyl acetate, and (Z,Z)-9,11-tetradecadienol confirmed that all three compounds are pheromone components. Subtraction of (Z,Z)-9,11-tetradecadienol from the blend completely eliminated its attractiveness, whereas the other two-component blends showed reduced activity. This is the first pheromone identification from the monotrysian superfamily Incurvarioidea, confirming that the common pheromones among ditrysian moths (long-chain fatty acid derivatives comprising alcohols, acetates, and aldehydes with one or more double bonds) is not an autapomorphy of Ditrysia, but a synapomorphy of the more advanced heteroneuran lineages.

Key Words—*Lampronia capitella*, Prodoxidae, Incurvarioidea, Lepidoptera, currant shoot borer, sex pheromone, EAG, GC-EAD, (Z,Z)-9,11-tetradecadienal, (Z,Z)-9,11-tetradecadienyl acetate, (Z,Z)-9,11-tetradecadienol.

INTRODUCTION

Larvae of several moth species may occur as pests in currant orchards. Economically most important are those species that infest the buds in early spring, destroying leaf and flower buds. In northern Europe, the currant shoot borer *Lampronia capitella* [Clerck, 1759] (Lepidoptera: Prodoxidae) is one of the major pests. *L. capitella* may be efficiently controlled by insecticide application in the early spring, before the larvae enter the buds. However, spraying operations are not always possible at this time of the year, because of problematic ground conditions. Alternatively, insecticides directed against the adults during their flight period might control the species. Identification of the sex pheromone of this species would greatly facilitate monitoring of the pest, and in integrated pest management, the pheromone could potentially be used for population control by mass trapping or mating disruption.

The identification of a sex pheromone from *L. capitella* would be of great phylogenetic interest as well. Börner (1925, 1939) proposed a fundamental division of Lepidoptera into Monotrysia and Ditrysia on the basis of the structure of the female genitalia. Ditrysia was correctly recognized as a natural monophyletic group that comprises approximately 95% of the Lepidoptera. In contrast, Monotrysia (*sensu* Börner) is not a monophyletic group, but contains all non-ditrysian moths. Sex pheromones have been identified from more than 400 species of ditrysian moths, whereas information on sex pheromones in non-ditrysian moths is limited (Löfstedt and Kozlov, 1997).

Sex pheromones of ditrysian moths are generally long-chain fatty acid derivatives (12–18 carbon atoms with one or two double bonds and hydroxyl (alcohols), acetoxy (acetates), or carbonyl (aldehydes) groups as terminal oxygen containing functions, released by females from glands located on the intersegmental membrane between the eighth and ninth abdominal segments (Percy-Cunningham and MacDonald, 1987; Arn et al., 1992). The females exhibit a more or less pronounced

calling posture in which the pheromone gland is exposed. In contrast, female sex pheromones have only been identified from four non-ditrysian species belonging to the families Nepticulidae and Eriocraniidae (Tóth et al., 1995; Zhu et al., 1995, Kozlov et al., 1996). In the eriocraniid moths, the pheromones are produced in sternum V glands, and moths from both families use short-chain alcohols and ketones as pheromones, distinctly different from the long-chain fatty acid derivatives identified from most ditrysian moths.

The superfamily Incurvarioidea to which the family Prodoxidae and the species *L. capitella* belong is one of the most advanced non-ditrysian families of moths. Thus, identification of pheromones from this family could shed light on the evolutionary origin of the typical ditrysian moth pheromones.

METHODS AND MATERIALS

Insects. L. capitella is a small (wingspan 14–17 mm) moth with holarctic (circumpolar) distribution (Northern and Central Europe, Siberia to Magadan, North America). Moths fly in June to early July.

The young larvae feed on seeds of young fruits of currant or gooseberry (*Ribes* spp.) until they leave the fruit and spin a strong hibernaculum low down on the currant bush among dead bud scales or in a crevice of the bark (Heath and Pelham-Clinton, 1976). After hibernation, in early spring, the larva enters a bud and consumes its leaves, flower buds, and pith. Immature larvae were collected in early May in Kannelmäki forest (Helsinki, Finland) where they fed on wild bushes of mountain currant, *Ribes alpinum*. Damaged twigs were cut and brought to the laboratory in Turku, where the larvae were reared until pupation, which usually took 3–7 d after collection. Pupation in this population occurs naturally in folded leaves, although in other populations it was reported to take place in a shoot (Heath and Pelham-Clinton, 1976). Pupae were sent to Lund and separated according to sex on the basis of genital characters. Adults were then allowed to emerge in an environmental chamber under a L14:D10 regime at 20°C and 60% RH.

Observation of Calling Behavior. Female moths were placed individually in 250 ml plastic cups covered with nylon screens and observed during a period of 4 d after emergence under the conditions described above. To roughly establish the calling period, observations were made at 1 hr intervals. Once the calling period had been ascertained, observations at 30 min intervals were made from 3 hr before to 3 hr after lights were turned on, and the number of calling females was recorded.

Preparation of Extracts. Insects were anesthesized by cooling them to −20°C. Head plus thorax, wings, the terminal abdominal segments (including the ovipositor), and the rest of the abdomen from 1–2 d-old female adults were dissected

by use of different scalpels for different body parts to avoid cross-contamination. Dissected parts were extracted for at least 30 min in laboratory-made microvials containing 50 μl hexane. These extracts were tested for EAG activity on male antennae. As a result of observations of the calling behavior of females and EAG tests, only female ovipositors were dissected and extracted for subsequent analyses.

Electrophysiological and Chemical Analyses. An excised male antenna was placed with the base in a capillary electrode filled with Beadle-Ephrussi Ringer solution and grounded via an Ag–AgCl wire. The distal tip of the antenna was placed in contact with a second electrode, similar to the indifferent electrode. The tip electrode was connected to a high-impedance dc amplifier with automatic baseline drift compensation. Extracts of different parts of the female body were tested for EAG activity by depositing one female equivalent inside the tip of a Pasteur pipette and blowing 1 ml of air through the pipette and onto the antenna. The activity of extracts was compared with the activity of 1 μg of synthetic samples of alcohols, identified as pheromone components of caddisflies or primitive moths. The alcohols tested in this experiment were racemic (*Z*)-6-nonen-2-ol, (2*R*,4*Z*)-hepten-2-ol and (2*R*)-heptan-2-ol (Zhu et al., 1995; Kozlov et al., 1996).

In another experiment, the EAG activity of all four geometric isomers of $\Delta 9,\Delta 11$-tetradecadienol and their corresponding acetates and aldehydes, altogether 12 different synthetic compounds, was investigated. Stimuli were prepared by applying hexane dilutions of the synthetic samples to pieces of filter paper in Pasteur pipettes. Very few insects were available for these experiments, and the low numbers did not allow replicated recordings. However, four different doses were tested for each compound (1, 10, 100, and 1000 ng). For each compound and dose, the relative response was calculated using the response to an air puff as control. A response 1.5 times as high as the response to the control was considered significant.

A Hewlett-Packard (H-P) 5890 Series II plus gas chromatograph (GC) equipped with either a polar HP Innowax column (30 m $\times$ 0.25 mm i.d., H-P) or a nonpolar DB-1 column (30 m $\times$ 0.25 mm i.d., J & W Scientific, Folsom, CA) and an effluent split allowed simultaneous flame ionization (FID) and electroantennographic detection (EAD) of the separated compounds. Hydrogen was used as carrier gas, and the effluent split ratio was approximately 1:1. Samples were injected splitless. The injector temperature was 250°C, and the split valve was opened 1 min after injection. The column temperature was maintained at 80°C for 2 min and then increased to 230°C at 10°C/min. The outlet for the EAD was placed in a purified humidified airstream flowing over the antennal preparation at a speed of 0.5 m/sec. Five microliters of an extract containing 2–3 female equivalents (FE) were injected onto the column for analysis. The equivalent chain lengths (ECLs) of EAD-active compounds were established relative to a homologous series of straight-chain acetates.

Preliminary GC–MS analyses of the GC-EAD active compounds were performed by using an H-P mass selective detector (MSD) interfaced with a 5890 series II GC. Final structure elucidation was performed with a VG 70-250 SE mass spectrometer (Vacuum Generators, Manchester, UK) linked to an H-P 5890 GC equipped with a 50 m $\times$ 0.25 mm i.d., 0.25 μm film thickness BPX5 column (SGE, Darmstadt, Germany) (injection at 60°C, 3 min hold, then 3°C/min to 300°C; splitless injection 1 min, 250°C; helium carrier gas). Chemical ionization mass spectrometry (CI-MS) to determine $M^+ + 1$ was carried out using isobutane as the reactant gas.

NMR spectra of the synthetic samples were run on an AMX-400 (Bruker; 400 MHz for ^{1}H and 101 MHz for ^{13}C) and DRX500 (Bruker; 500 MHz for ^{1}H and 126 MHz for ^{13}C) instrument.

Field Tests. Synthetic blends were prepared in hexane. Red rubber septa (Arthur Thomas Co., Catalog No. 1780-J07) were used as dispensers, and the traps used were laboratory-made "Delta traps". Field trapping was conducted in an old black currant orchard in Sörfors (63°52″N, 20°01″E) close to Umeå in northern Sweden, between June 18 and July 10, 1998. All traps were hung on black currant branches 1–1.5 m above ground. Within a replicate ($N = 5$), traps were set at least 3 m apart. To minimize the effects of habitat heterogenities, trap positions were randomized within a replicate. Traps were moved on one position after each check. Dispensers were renewed on June 29. Traps were checked five times during the trapping period. Statistical significance of the results was tested by one-way ANOVA of square root-transformed data followed by multiple comparisons according to the least significant difference method.

Synthesis of Reference Compounds

(9Z, 11Z)-Compounds. Commercially available 8-bromooctan-1-ol (Aldrich) was protected as the tetrahydropyranyl derivative and alkylated with propynol to yield 11-(tetrahydropyran-2-yloxy)-2-undecyn-1-ol. The synthesis was similar to that described previously by Ochiai et al. (1983) except that THF:HMPT (1:1) was used instead of pure HMPT, and the coupling reaction was carried out at −30°C followed by slow warming to room temperature. After workup, the corresponding propargyl alcohol was obtained in 67% yield. This alcohol was oxidized with pyridinium dichromate in dichloromethane and converted to 2-[(11*Z*)-tetradec-11-en-9-ynyl-1-oxy]-tetrahydro-2*H*-pyran by a (*Z*)-selective Wittig-reaction with *n*-propyl triphenylphosphonium bromide (Aldrich) using sodium bis-(trimethylsilyl) amide as base (Bestmann et al., 1976). The reaction was carried out in THF during 30 min at –20°C and completed upon stirring for another 30 min at +20°C. The final product (82% yield) contained 9% of the (*E*)-isomer. A (*Z*)-selective reduction

of the triple bond was achieved by using dicyclohexylborane in hexane (Wong et al., 1984). Removal of the tetrahydropyranyl group under acidic conditions (*p*-toluenesulfonic acid, PTSA, in MeOH) yielded (9*Z*,11*Z*)-tetradecadienol. This was acetylated with acetic anhydride in pyridine to afford (9*Z*,11*Z*)-tetradecadienyl acetate or oxidized using the Swern method (Mancuso and Swern, 1981) to afford (9*Z*,11*Z*)-tetradecadienal. The final products (alcohol, acetate, and aldehyde) contained 9% of the corresponding (9*Z*,11*E*)-isomers.

(9E,11E)-Compounds. The (9*E*,11*E*)-compounds were prepared according to the general approach described by Samain and Descoins (1978). The (9*E*,11*E*)-tetradecadienol so obtained contained 14% of the (9*E*)-8-ethyldodeca-9,11-dienol (as observed by Burger et al., 1990), and the same amount of a corresponding contamination was found in the acetate and aldehyde. Each of the three final products showed an isomeric purity of about 98%.

(9Z,11E)-Compounds. (9*Z*,11*E*)-Tetradecadien-1-ol is commercially available (Sigma). Acetylation or Swern oxidation yielded the corresponding acetate and aldehyde. The final products contained 2% of the (9*Z*,11*Z*)-isomers and 2% of the (9*E*,11*E*)-isomers.

(9E,11Z)-Compounds. Synthesis of the (9*E*,11*Z*)-tetradecadienyl compounds started with 11-(tetrahydropyran-2-yloxy)-2-undecyn-1-ol (see above). The triple bond was (*E*)-selectively reduced with lithium aluminum hydride in THF (Rossi and Carpita, 1977). Subsequent oxidation with pyridinium dichromate in dichloromethane yielded (2*E*)-11-(tetrahydropyran-2-yloxy)-2-undecenal. Wittig reaction with the ylide from *n*-propyl triphenylphosphonium bromide was carried out (*Z*)-selectively as described above. Removal of the tetrahydropyranyl group under acidic conditions (PTSA in MeOH) yielded (9*E*,11*Z*)-tetradecadien-1-ol. This was acetylated with acetic anhydride in pyridine to afford (9*E*,11*Z*)-tetradecadienyl acetate. Swern oxidation (Mancuso and Swern, 1981) afforded (9*E*,11*Z*)-tetradecadienal. The final products contained 8% of the (9*E*,11*E*)-isomers.

Spectroscopic Data of Synthetic Samples

Spectroscopic data of alcohols and acetates matched those reported in the literature (Bestmann et al., 1981; Björkling et al., 1987). To our knowledge, no comprehensive compilation of data on the aldehydes is available in the literature and thus we include the spectral data of the following.

(9Z,11Z)-Tetradecadienal. ^{1}H NMR (400 MHz, $CDCl_3$): $\partial = 1.00$ (t, 3H, $J = 7.6$ Hz, 14-H), 1.25–1.41 (m, 8H, 4-H to 7-H), 1.58–1.67 (m, 2H, 3-H), 2.11–2.23 (m, 4H, 8-H and 13-H), 2.42 (dt, 2H, $J = 7.3$, 2.0 Hz, 2-H), 5.40–5.49 (m, 2H, 9-H and 12-H), 6.16–6.29 (m, 2H, 10-H and 11-H), 9.76 (t, 1H, $J = 2.0$ Hz, 1-H) (see also Ramiandrasoa and Tellier, 1990).

^{13}C NMR (126 MHz, $CDCl_3$): $\partial = 14.20$ (q, 14-C), 20.81/22.07/27.40/29.02/29.12/29.20/29.54 (7t, 3-C to 8-C and 13-C), 43.91 (t, 2-C), 122.99/123.59 (2d, 9-C and 12-C), 131.94/133.72 (2d, 10-C and 11-C), 202.85 (d, 1-C).

MS (70 eV) m/z (%): 208 (2, M^+), 152 (0.4), 151 (0.7), 137 (0.7), 135 (1), 121 (1), 110 (2), 109 (3), 98 (4), 96 (5), 95 (19), 93 (7), 91 (7), 82 (20), 81 (19), 79 (25), 77 (14), 68 (18), 67 (65), 65 (10), 57 (10), 55 (34), 53 (20), 43 (37), 41 (100), 39 (57).

(9E,11Z)-Tetradecadienal. ^{1}H NMR (500 MHz, $CDCl_3$): $\partial = 0.99$ (t, 3H, $J = 7.4$ Hz, 14-H), 1.22–1.42 (m, 8H, 4-H to 7-H), 1.59–1.66 (m, 2H, 3-H), 2.09 (q, 2H, $J = 7.5$ Hz, 8-H), 2.18 (m, 2H, $J = 7.5$, 1.26 Hz, 13-H), 2.42 (dt, 2H, $J = 7.4$, 1.9 Hz, 2-H), 5.30 (dt, 1H, $J = 10.7$, 7.6 Hz, 12-H), 5.65 (dt, 1H, $J = 15.1$, 6.9 Hz, 9-H), 5.91 (dd, 1H, $J = 11.0$, 10.7 Hz, 11-H), 6.30 (ddq, 1H, $J = 15.1$, 11.0, 1.26 Hz, 10-H), 9.76 (t, 1H, $J = 1.9$ Hz, 1-H).

^{13}C NMR (126 MHz, $CDCl_3$): $\partial = 14.31$ (q, 14-C), 21.01/22.07/28.98/29.10/29.20/29.30/32.81 (7t, 3-C to 8-C and 13-C), 43.90 (t, 2-C), 125.60/128.00 (2d, 9-C and 12-C), 131.74/134.51 (2d, 10-C and 11-C), 202.88 (d, 1-C).

MS (70 eV) m/z (%): 208 (8, M^+), 152 (1), 151 (3), 137 (2), 135 (4), 121 (6), 110 (5), 109 (20), 98 (8), 96 (10), 95 (37), 93 (12), 91 (10), 82 (32), 81 (29), 79 (26), 77 (13), 68 (31), 67 (100), 65 (10), 57 (7), 55 (44), 53 (19), 43 (15), 41 (49), 39 (17).

(9E,11E)-Tetradecadienal. ^{1}H NMR (500 MHz, $CDCl_3$): $\partial = 1.00$ (t, 3H, $J = 7.2$ Hz, 14-H), 1.24–1.41 (m, 8H, 4-H to 7-H), 1.58–1.66 (m, 2H, 3-H), 2.01–2.11 (m, 4H, 8-H and 13-H), 2.41 (dt, 2H, $J = 7.6$, 1.9 Hz, 2-H), 5.52–5.65 (m, 2H, 9-H and 12-H), 5.96–6.03 (m, 2H, 10-H and 11-H), 9.76 (t, 1H, $J = 1.9$ Hz, 1-H).

^{13}C NMR (126 MHz, $CDCl_3$): $\partial = 13.64$ (q, 14-C), 22.07/25.59/28.94/29.11/29.21/29.34/32.54 (7t, 3-C to 8-C and 13-C), 43.91 (t, 2-C), 129.37/130.45 (2d, 9-C and 12-C), 132.25/133.95 (2d, 10-C and 11-C), 202.87 (d, 1-C).

MS (70 eV) m/z (%): 208 (14, M^+), 152 (1), 151 (2), 137 (2), 135 (4), 121 (4), 110 (4), 109 (8), 98 (10), 96 (15), 95 (57), 93 (18), 91 (11), 82 (50), 81 (39), 79 (34), 77 (12), 68 (30), 67 (100), 65 (7), 57 (4), 55 (31), 53 (19), 43 (15), 41 (49), 39 (10) (see also Ando et al., 1988).

(9Z,11E)-Tetradecadienal. ^{1}H NMR (500 MHz, $CDCl_3$): $\partial = 1.02$ (t, 3H, $J = 7.5$ Hz, 14-H), 1.24–1.40 (m, 8H, 4-H to 7-H), 1.59–1.66 (m, 2H, 3-H), 2.09–2.19 (m, 4H, 8-H and 13-H), 2.42 (dt, 2H, $J = 7.6$, 1.9 Hz, 2-H), 5.29 (dt, 1H, $J = 10.7$, 7.6 Hz, 9-H), 5.70 (dt, 1H, $J = 15.1$, 6.6 Hz, 12-H), 5.95 (dd, 1H, $J = 11.0$, 10.7 Hz, 10-H), 6.28 (ddq, 1H, $J = 15.1$, 11.0, 1.3 Hz, 11-H), 9.76 (t, 1H, $J = 1.9$ Hz, 1-H) (see also Ramiandrasoa and Tellier, 1990).

^{13}C NMR (126 MHz, $CDCl_3$): $\partial = 13.67$ (q, 14-C), 22.07/25.89/27.62/29.00/29.12/29.21/29.62 (7t, 3-C to 8-C and 13-C), 43.90 (t, 2-C), 124.66/128.70 (2d, 9-C and 12-C), 129.96/136.23 (2d, 10-C and 11-C), 202.89 (d, 1-C).

MS (70 eV) m/z (%): 208 (6, M^+), 152 (2), 151 (2), 137 (1), 135 (3), 121 (3), 110 (4), 109 (14), 98 (8), 96 (10), 95 (40), 93 (12), 91 (9), 82 (38), 81 (33), 79 (26), 77 (11), 68 (30), 67 (100), 65 (8), 57 (1), 55 (36), 53 (14), 43 (12), 41 (45), 39 (15).

RESULTS

Calling Behavior. Females extended their terminal abdominal segments in a typical moth calling behavior. Female calling began when lights were turned on and peaked 60 min after the beginning of the photoperiod (75% of the females calling). Calling had ceased 150 min into the photoperiod, and few females older than 3 d were observed calling.

EAG Tests of Extracts from Different Body Parts and Synthetic Reference Compounds. Extracts of the terminal abdominal segments showed highest EAG acitivity. Extracts of the rest of the abdomen as well as other body parts also showed significant but much lower activity, whereas the compounds previously identified from caddisflies and primitive Lepidoptera were no more active than the solvent when tested in this experiment (Figure 1).

GC-EAD of Female Extracts and Chemical Analyses. GC-EAD analyses of the extracts from the terminal abdominal segment demonstrated that three

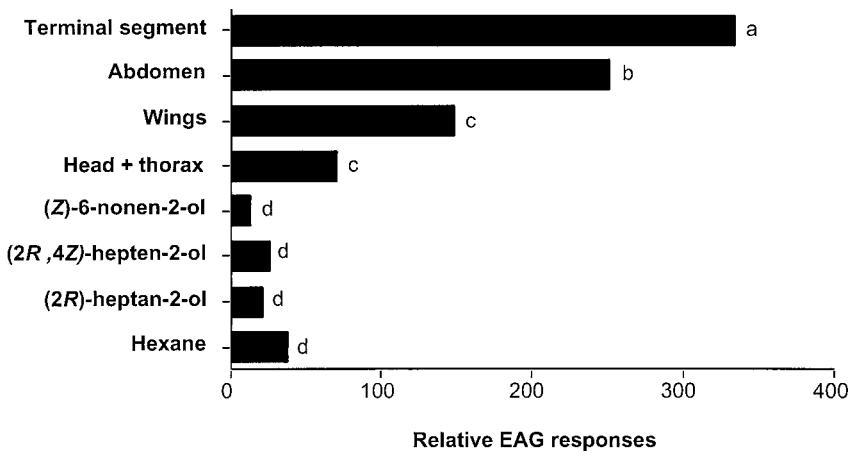

FIG. 1. Relative electroantennogram responses of antennae of male *Lampronia capitella* to extracts of different body parts of conspecific females, and synthetic pheromone components previously identified from monotrysian moths. Bars followed by the same letter are not significantly different (Kruskal–Wallis analysis of variance followed by pairwise comparisons using a Mann–Whitney U-test ($P > 0.05$)).

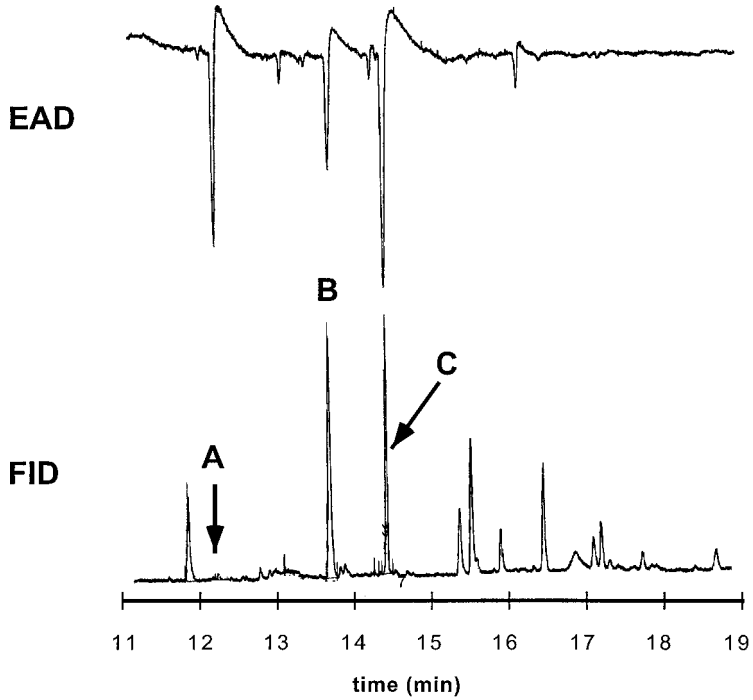

FIG. 2. Simultaneously recorded flame ionization detector (FID) and electroantennographic detector (EAD) responses using antennae of male *Lampronia capitella* in response to pheromone gland extracts from conspecific females. An Innowax column was used for separation.

compounds (A, B, and C) elicited strong responses from an antenna of a conspecific male when analyzed on an Innowax column (Figure 2). The equivalent chain lengths (ECLs) of the three pheromone candidates were 1443, 1598, and 1681, respectively. Preliminary GC–MS analysis revealed that active compound B most likely was a minor compound coeluting with a large amount of tricosane on the Innowax column. Thus, new samples were subjected to GC-EAD analysis using a nonpolar DB-1 column. Again, three active compounds (this time called compounds I–III) were consistently detected. The ECL values of these compounds were calculated to be 1243, 1317, and 1443 on the nonpolar column.

Relative amounts of the three compounds varied among extracts but compound II was always most abundant (0.5–10 ng per female equivalent) and compounds I and III were in the range of 5–25% of the major compound.

Structure Elucidation. Upon analysis on the nonpolar column, compound III gave a particularly informative mass spectrum, showing a molecular

ion at $M^+ = 252$ in the EI mode (confirmed as m/z 253 $= M^+ + 1$ upon CI). Key fragments in the EI spectrum at m/z 61 (protonated acetic acid) and m/z 192 ($M^+ -$ acetic acid; $C_{14}H_{24}$ upon high resolution MS) revealed that III might be the acetate of a tetradecadienol. A rather abundant molecular ion in the EI spectrum pointed to the presence of a conjugated double bond system (Löfstedt and Odham, 1984). The general fragmentation pattern and the relative intensities of some fragments in the lower mass region, especially m/z 95 and m/z 82, indicated the double bonds to be in the 9,11-position (Ando et al., 1988). The stereochemistry of the double bond system was determined by comparison of retention times of the natural product and synthetic samples. The four stereoisomers of 9,11-tetradecadienyl acetate were well separated on the nonpolar column: (9*Z*,11*E*)-isomer eluted at 50 min 6 sec, and (9*E*,11*Z*)-isomer at 50 min 26 sec, (9*Z*,11*Z*)-isomer at 50 min 42 sec, and (9*E*,11*E*)-isomer at 50 min 53 sec. Upon coinjection, (9*Z*,11*Z*)-tetradecadienyl acetate coeluted with the natural product and showed an identical mass spectrum.

Compound II, the most abundant EAD-active compound with an ECL of 1317 on the nonpolar column, showed a slight tailing under the chromatographic conditions used. The abundant molecular ion of $M^+ = 210$ (confirmed as m/z 211 $= M^+ + 1$ upon CI), a fragment at m/z 192 (loss of water), and the similarity of the fragmentation as compared to compound III, strongly suggested compound II to be (9*Z*,11*Z*)-tetradecadienol. A synthetic sample (like the acetate, eluting third in the mixture of stereoisomers) had the same retention time as the natural product and yielded the identical mass spectrum.

Compound I showed an ECL of 1243 on the nonpolar column and a molecular ion at m/z 208. A small signal at m/z 58 suggested a carbonyl group, and from the fragmentation it could be concluded that this compound was the aldehyde corresponding to II and III. Mass spectra of compounds I, II, and III showed the same base peak at m/z 67 and abundant fragments at m/z 81 and 82 as well as m/z 95. Synthetic (9*Z*,11*Z*)-tetradecadienal had the same retention time as compound I and showed an identical mass spectrum.

No insect-derived material was available for coinjection with synthetic samples on the Innowax column. However, the ECL values of the aldehydes, acetates, and alcohols used as references were determined and compared with the ECL values of the EAD-active peaks obtained 1 year before on a column with the same stationary phase. The isomers of all three compound classes eluted in the order *ZE*, *EZ*, *ZZ*, and *EE*. The closest match was in all three cases obtained with the *ZZ* isomer: *Z*9,*Z*11-14:Ald (1437), *Z*9,*Z*11-14:OAc (1596), and *Z*9,*Z*11-14:OH (1677) to be compared with compound A (1443), compound B (1598), and compound C (1681).

EAG Screening of Synthetic Tetradecadienyl Compounds. At the lowest dose (1 ng), only the *ZZ* and the *ZE* aldehydes elicited significant reponses, the *ZZ* aldehyde being most active. At the highest dose (1000 ng), all tested compounds

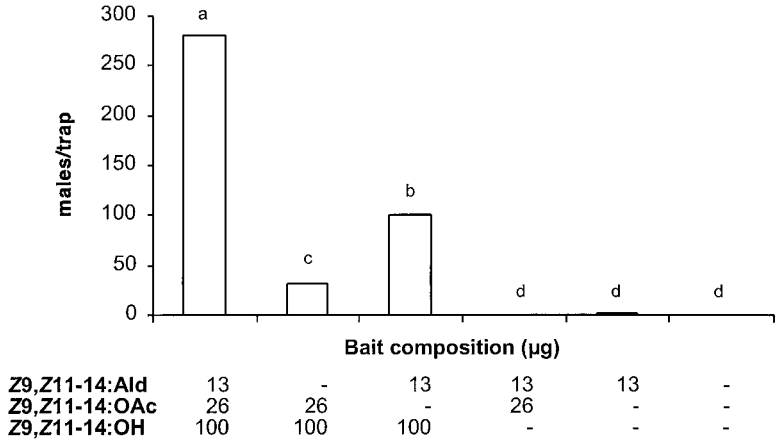

FIG. 3. Field catches of male *Lampronia capitella* in traps baited with various combinations of pheromone component candidates. Treatments labelled with the same letters are not significantly different (one-way ANOVA of square root-transformed data followed by multiple comparisons according to the least significant difference method ($P < 0.01$).

generated significant responses, the *ZZ* aldedyde being the most active (relative response 17.1) and the *EE* acetate being the least active compound (relative response 2.1). The order of relative activities for the 12 compounds was (listed from highest to lowest) *ZZ* aldehyde, *ZE* aldehyde, *EE* aldehyde, *ZZ* alcohol, *ZE* alcohol, *EZ* aldehyde, *ZZ* acetate, *EE* alcohol, *ZE* acetate, *EZ* alcohol, *EZ* acetate, and *EE* acetate.

Field Tests. The synthetic mixture of the three compounds identified from female extracts trapped large numbers of conspecific males, whereas the attraction to unbaited control traps was virtually zero (Figure 3). Subtraction of any of the compounds from the three-compound mixture reduced trap catch significantly. Subtraction of the alcohol completely eliminated the attractiveness of the blend, whereas two-compound blends lacking aldehyde or acetate still showed significant but reduced attractiveness. The aldehyde, being the most EAG-active compound, was the only compound tested as a single compound, but it was not attractive as such.

DISCUSSION

The female-produced sex pheromone of *L. capitella* was identified as a mixture of *Z*9,*Z*11-14:OH and the corresponding aldehyde and acetate. This is the first pheromone identification from the monotrysian superfamily Incurvarioidea and it confirms that the common pheromone component type among ditrysian

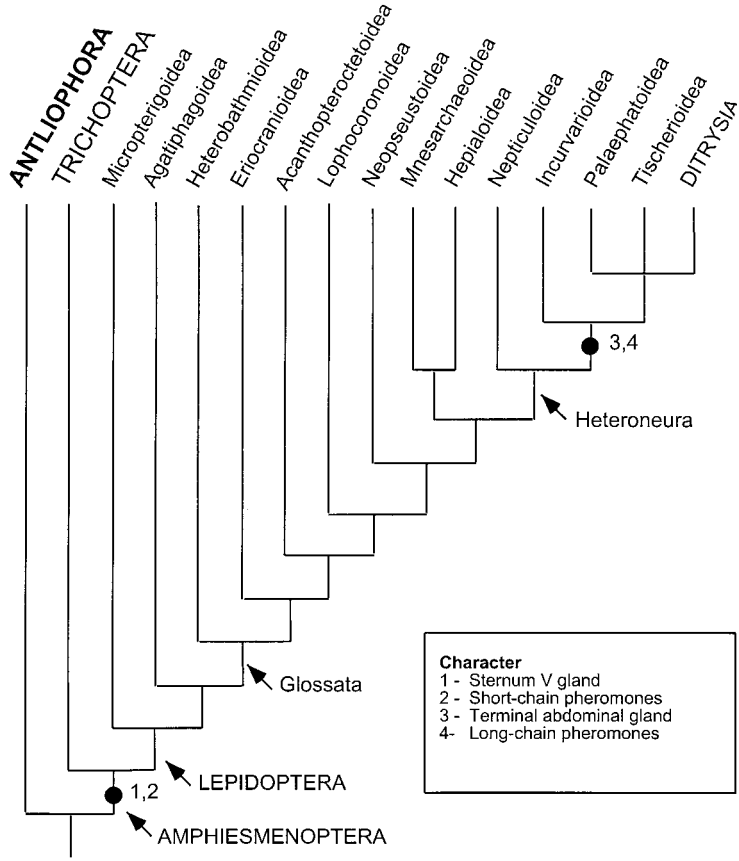

FIG. 4. Cladogram of the basic lineages of the panorpoid insects and of Lepidoptera (modified from Löfstedt and Kozlov, 1997). Sequence of lineages after Kristensen (1984) and Kristensen and Skalski (1999). Filled circles are apomorphic character states (derived state relative to another state).

moths (long-chain fatty acid derivatives comprising alcohols, acetates, and aldehydes with one or more double bonds) is not an autapomorphy* of Ditrysia, but a synapomorphy[8] of the more advanced heteroneuran lineages (Figure 4) (Löfstedt and Kozlov, 1997).

*Z*9,*Z*11-14:OAc was previously identified as a pheromone component in the geometrid moth *Idaea aversata* (Zhu et al., 1996) and as a pheromone gland

*Within the context of a particular group of organisms, an *apomorphy* is a character state derived within the group. A character state shared by members of a group is a *synapomorphy* of the members of the group (postulated to have evolved in their common ancestor) and an *autapomorphy* of the group.

compound in *Spodoptera littoralis* (Dunkelblum et al., 1982). The shorter homologue (*Z*,*Z*)-7,9-dodecadienyl acetate has been identified from moths belonging to the Noctuidae, Geometridae, Pyralidae, Pterophoridae, Tortricidae, and Elachistidae families (http://www-pherolist.slu.se/, an updated website based on the book of Arn et al., 1992). *Z*9,*Z*11-14:OH and *Z*9,*Z*11-14:Ald were reported as attractants for *Leptosteles ferruminaria* (Reed and Chisholm, 1985).

The (*Z*,*Z*)-9,11-tetradecadienyl pheromone components were identified on the basis of mass spectra and GC retention times. The assignments were corroborated by the EAG data on synthetic compounds and the strong attraction to the synthetic blends in the field. Mass spectra (70 eV EI) of many conjugated tetradecadienyl compounds show characteristic fragmentation (Ando et al., 1988) although relative intensities of diagnostic signals may show some variation between instruments. The fragmentation pathways lead to dominant fragment ions of m/z 82 and 95 exclusively in case of 9,11-tetradecadienyl compounds, which were also dominant in the mass spectra of the three EAD-active *Lampronia* components. Especially the ($m/z = 82$:$m/z = 81$) > 1—ratio of these spectra seems to be typical for such structures because ($m/z = 82$:$m/z = 81$) < 1 for all other reported tetradecadienyl compounds. More detailed EI mass spectra of 9,11-tetradecadienyl acetates (Rossi et al., 1981; Cuvigny et al., 1987) also showed a good agreement with the analogous *Lampronia* component.

The sequence of elution of the four geometric acetate isomers on the polyethylene glycol-type stationary phase (Innowax) was the same as the one obtained by Zhu et al. (1996). We found the same elution order for the alcohols and the aldehydes. On the Innowax column, the *Z*9,*Z*11-14:OAc coeluted with tricosane. The insect extract contained large amounts of this hydrocarbon, and as a result the acetate could not be unambiguously identified using the Innowax column. In all cases, the natural 9,11-tetradecadienes of *L. capitella* were resolved from other components on the nonpolar column, and coeluted with the reference (*Z*,*Z*)-isomers.

In the EAG screening of the synthetic compounds, aldehydes were generally more active than the alcohols with acetates being the least active. However, it should be noted that aldehydes were the most volatile of the compounds and, thus, the amount actually hitting the antenna was higher for the aldehydes than for the other compound classes. For all compound classes, the (*Z*,*Z*)-isomer was the most active, supporting the chemical identification of the double bond geometry.

The synthetic compounds used in field trapping contained approximately 9% of the corresponding (*Z*,*E*)-isomers. These impurities could either synergize or reduce the attraction to the (*Z*,*Z*)-isomers, or they may have no effect. The synthesis of (*Z*,*Z*)-isomers of high purity is not trivial and even pure samples may easily isomerize. For practical purposes, it would be of interest to optimize the synthetic pheromone for maximum attraction and to investigate the effect of isomeric impurities on the activity. Optimization of the synthetic pheromone also should include optimization of the ratio between the compounds and the dose.

It appears likely that the doubly unsaturated pheromone components identified from *L. capitella* are biosynthetically closely related to other common moth pheromone compounds, including structures such as (*E*)-11-tetradecenyl acetate, (*Z*)-11-tetradecenyl acetate, (*Z*)-9-tetradecenyl acetate, (*Z*)-7-dodecenyl acetate, (*Z*)-11-hexadecenyl acetate, and their corresponding aldehydes and alcohols. These structures can all be derived from palmitic acid by the combined action of Δ11 desaturases and chain shortening enzymes. Furthermore, the *L. capitella* pheromone is produced in a terminal abdominal gland and females emitting pheromone display a calling behaviour similar to that reported for many ditrysian moths. This suggests that the typical moth pheromone components and the terminal glands evolved in the early Heteroneura, before the divergence of Ditrysia and other heteroneuran lineages (Figure 4).

The synthetic three-component pheromone reported here is an effective and specific attractant for *L. capitella* that can be used for monitoring. We found no other moth species in any significant numbers in our traps. To allow the use of insecticides to be decreased, a monitoring bait should be developed for *Euhyponomeutoides albithoracellus* (earlier *Kessleria rufella*) [Gaj, 1954] (Lepidoptera: Yponomeutidae) as well, the other major moth pest in currant orchards in northern Europe. Mating disruption with synthetic pheromone may be attempted against *L. capitella* because this technique has produced successful population control with many other pests in vineyards and fruit orchards (see e.g., Arn and Louis, 1997; Gut and Brunner, 1999).

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(2*R*,7*S*)-DIACETOXYTRIDECANE: SEX PHEROMONE OF THE APHIDOPHAGOUS GALL MIDGE, *Aphidoletes aphidimyza*

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Abstract—In a recent study, evidence was presented that females of the aphidophagous midge *Aphidoletes aphidimyza* (Rondi) (Diptera: Cecidomyiidae) release a sex pheromone to attract mates. Our objectives were to identify and bioassay the pheromone. Coupled gas chromatographic–electroantennographic detection (GC-EAD) analyses of untreated and hydrogenated pheromone extract on three fused-silica columns (DB-5, DB-23, DB-210) revealed a single compound that elicited responses from male antennae. Retention index calculations of this candidate pheromone (CP) suggested that it was a di-acetate. Considering that most of the presently identified cecidomyiid pheromones consist of a 13-carbon chain with (at least) one acetate group in C2, we synthesized 2,6-, 2,7-, 2,8-, 2,9-, 2,10-, 2,11-, and 2,12-diacetoxytridecane. In GC analyses of these compounds, only 2,7-diacetoxytridecane cochomatographed with CP on all columns. In laboratory two-choice experiments with stereospecifically synthesized stereoisomers, only (2*R*,7*S*)-diacetoxytridecane elicited significant anemotatic responses by male *A. aphidimyza*. In trapping experiments in greenhouse compartments, only traps baited with (2*R*,7*S*)-diacetoxytridecane captured significant numbers of male *A. aphidimyza*, clearly revealing the absolute configuration of the pheromone. Failure of the stereoisomeric mixture (containing all four stereoisomers including the pheromone) to attract males is due

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to inhibitory characteristics of the (2*R*,7*R*)- and (2*S*,7*R*)-stereoisomers. The pheromone of zoophagous *A. aphidimyza* resembles those from phytophagous cecidomyiid midges, suggesting a common, diet-independent pathway for pheromone biosyntheses.

Key Words—*Aphidoletes aphidimyza*, Cecidomyiidae, sex pheromone, stereoisomers, (2*R*,7*S*)-diacetoxytridecane, (2*S*,7*R*)-diacetoxytridecane, (2*R*,7*R*)-diacetoxytridecane, (2*S*,7*S*)-diacetoxytridecane.

INTRODUCTION

Gall midge larvae have diverse feeding habits, including phytophagy, mycetophagy, and zoophagy (Barnes, 1956). Larvae of *Aphidoletes aphidimyza* (Rondi) (Diptera: Cecidomyiidae) are zoophagous and prey upon most true aphids. In commercial greenhouses, inoculative or inundative release of *A. aphidimyza* is used to control several aphid species on crops such as sweet pepper, *Capsicum annuum* L., and tomato, *Lycopersicon esculentum* L. (van Lenteren and Woets, 1988; Rabasse and van Steenis, 1999; van Schelt and Mulder, 2000).

Sexual communication and mating behavior of *A. aphidimyza* are spectacular (van Lenteren et al., 2002). At night, females seek sticky spider webs, possibly guided by spider pheromones shown to emanate from webs (Schultz and Toft, 1993). Shortly after landing on webs, female *A. aphidimyza* start calling by extending and slowly moving the terminal part of the abdomen (van Lenteren et al., 2002). They release pheromone from glandular epithelium of the 8th–9th intersegmental membrane (van Lenteren et al., 2002), a gland similar to those described in other Cecidomyiidae (Solinas and Isodoro, 1991, 1996; Isodoro et al., 1992). The pheromone mediates long-range attraction of conspecific males, as demonstrated in wind tunnel experiments with virgin, mated, and ovipositor-ectomized virgin females (van Lenteren et al., 2002).

There are many mysteries in the chemical ecology of *A. aphidimyza* including adaptations that enable these midges to land on and leave sticky spider webs without getting caught, and the communication signal(s) that they utilize to attract mates. We report identification, synthesis, and testing of the female-produced sex pheromone.

METHODS AND MATERIALS

Experimental Insects and Acquisition of Pheromone Extract. Pupae of *A. aphidimyza* were obtained from Applied Bio-nomics Ltd. (Sidney, British Columbia, Canada V8L 5P5) and kept in the Insectary at Simon Fraser University at a temperature of 23 $\pm$ 1.5°C and a photoperiod of L16: D8. Eclosed adults were collected at 1-hr intervals during the photophase, separated by sex based on

antennal dimorphism, and placed in separate Plexiglass containers (15 × 15 × 15 cm). Insects were sustained with a 20% sucrose solution dispensed from a tissue paper wick inside a 20-ml vial. Abdominal tips with pheromone glands (van Lenteren et al., 2002) of 1–3-d-old calling females were removed during the first 5–180 min of the scotophase and extracted in pentane. The supernatant was withdrawn, transferred into a vial, and stored at −20°C.

Laboratory Analysis of Pheromone Extract and General Instrumentation. Pheromone gland extracts were analyzed by coupled gas chromatographic-electroantennographic detection (GC-EAD) and GC-mass spectrometry (MS), and candidate pheromones were synthesized using procedures and equipment as previously described in detail (Gries et al., 2002).

Syntheses

*2,7-Diacetoxytridecane (Compound **4**, Scheme 1).* 1,4-Dichlorobutane (1.65 ml, 15 mmol) and 1.1 g of Mg (45 mmol) were refluxed in 50 ml of THF for 3 hr until the Grignard reagent had formed completely. The solution was cooled to 0°C, and a mixture of 1.12 ml (20 mmol) of acetaldehyde and 2.80 ml (20 mmol) of heptanal in 30 ml of THF was added dropwise. The reaction mixture was stirred for another 30 min while warming to room temperature, and then quenched with conc. aq. NH_4Cl. Reaction products were extracted with ether (3 × 75 ml), extracts washed with brine and dried (anh. $MgSO_4$), and solvents evaporated in vacuo. The resulting diols **1**, **2**, and **3** (1.1 : 2 : 0.9; on the basis of GC analysis) (Scheme 1) were separated (eluting in reverse order) by flash chromatography (50 g of silica), using hexane/ether as eluent with a gradual increase (15%–50%) of the ether proportion. Chromatography afforded 1.30 g of **2** (93% pure, 5.63 mmol, 75% yield based on statistical distribution of diols, 38% yield based on dichlorobutane). Overnight acetylation of 0.40 g (1.73 mmol) of **2** with excess of acetic anhydride (1 ml) and pyridine (3 ml) afforded after workup and column purification 0.415 g of the diacetoxytridecane **4** (1.38 mmol, 80% yield). Anal. calcd. for $C_{17}H_{32}O_4$: C 67.96; H 10.73. Found: C 68.01; H 10.78. ^{1}H NMR: $CDCl_3$, δ = 4.80–4.92 (2H, m), 2.03 (3H, s), 2.02 (3H, s), 1.45–1.55 (6H, m), 1.21–1.35 (12H, m), 1.19 (3H, d), 0.88 (3H, t); GC-MS [*m*/*z* (relative intensity)]: 41 (19), 43 (100), 55 (20), 67 (37), 81 (24), 95 (84), 97 (37), 111 (31), 113 (29), 125 (21), 180 (4), 197 (8). Other diacetoxytridecanes, such as 2,6-, 2,7-, 2,8-, and 2,11-diacetoxytridecane, were synthesized by: (a) coupling of corresponding α,ω-di-Grignard reagents with a mixture of the two aldehydes (as in Figure 1, Scheme 1); (b) separation of the diols; and (c) acetylation of target asymmetric diols.

Stereoselective Syntheses. The four individual stereoisomers of 2,7-diacetoxytridecane (**4**) (*RR*-, *RS*-, *SR*-, and *SS*-**4**) were produced analogous to syntheses of the four stereoisomers of 2,7-nonanediyl dibutyrate (Gries et al., 2000),

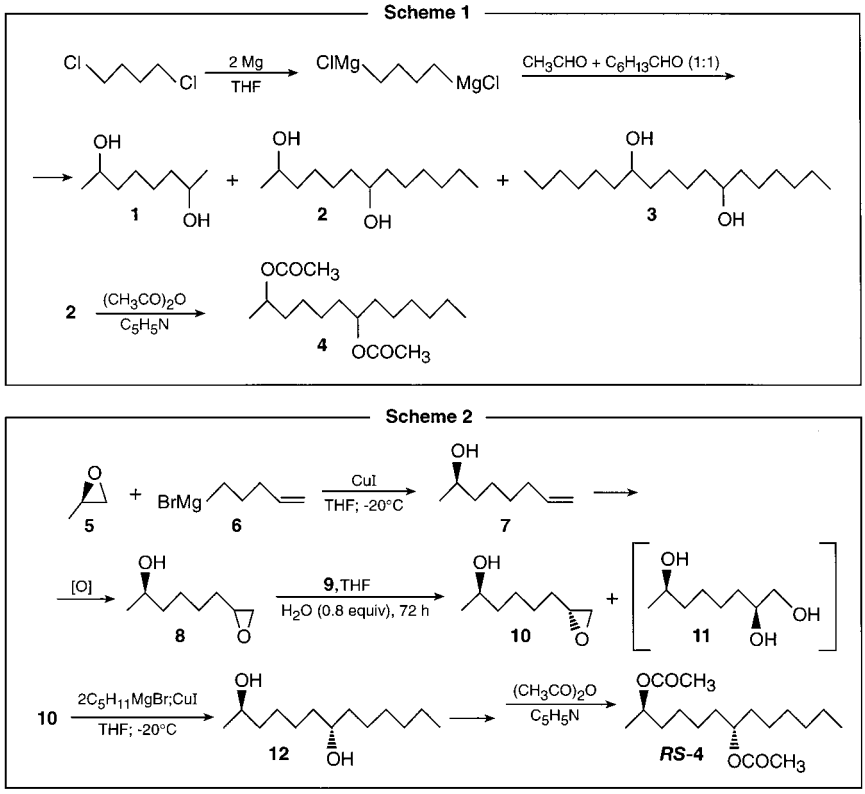

FIG. 1. Syntheses of stereoisomeric 2,7-diacetoxytridecane (**4**, Scheme 1) and (2*R*,7*S*)-diacetoxytridecane (***RS*-4**, Scheme 2).

as follows: (a) opening of the (*R*)- or (*S*)-propyleneoxide by 4-penten-1-yl magnesium bromide; (b) epoxidation of the resulting secondary alcohols by *m*-chloro-perbenzoic acid; (c) hydrolytic kinetic resolution (HKR) of the two terminal epoxides with the (*R*,*R*)- or (*S*,*S*)-Co(II) salen (Jacobsen's) catalyst (Tokunaga et al., 1997; Schaus et al., 1998; Gries et al., 2000), yielding the four stereoiomers of 1,2-epoxy-7-hydroxyoctanes [synthesis of the (*R*,*S*)-stereoisomer **10** shown in Figure 1, Scheme 2]; and (d) opening of the epoxy ring of these synthetic intermediates with amylmagnesium bromide, and subsequent acetylation, affording the target *RR*-, *RS*-, *SR*-, and *SS*-**4** [synthesis of *RS*-**4** shown in Scheme 2]. Prolonged exposure (up to 72 hr) of the intermediate hydroxyepoxides to HKR, and increasing amounts of water [from 0.55 equivalents (Schaus et al., 1998) to 0.85 equivalents] improved enantiomeric excess (ee) of final products from ~75–80% to >98% without compromising yield significantly.

Enantiomeric excess (ee) was determined by GC with a Cyclodex-B column (30 m × 0.25 mm ID; J&W Scientific, Folsom, California, USA; chromatography: 120°C isothermal), which separated *SS*-**4** (296 min) and *SR*-**4** (306 min) with baseline resolution, and *RR*-**4** (323 min) and *RS*-**4** (328 min) with near-baseline resolution. All test chemicals were >95% chemically pure; enantiomeric excess of the *RR*-, *RS*-, *SR*-, and *SS*-**4** was 98.6, 99.0, 85.8, and 94.6%, respectively (Table 1).

*(2R,7S)-Diacetoxytridecane (Compound **RS-4** in Scheme 2).* (*R*)-Propylene oxide (**5**) (2 ml, 28.6 mmol) was coupled at −20°C with 40 mmol of 1-pentene-5-yl magnesium bromide (**6**) in the presence of 4 mmol (0.76 g) of catalytic CuI. After 1 hr stirring, the reaction mixture was warmed to 0°C and quenched with conc. aq. NH_4Cl. Usual workup and column purification afforded (2*R*)-7-octen-2-ol (**7**) (97% pure by GC) with near quantitative yield. Alcohol **7** was oxidized with excess of *m*-chloro-perbenzoic acid (9.6 g, 43 mmol, 77% pure) in dichloromethane for 5 hr at 0°C, and then for 2 hr at room temperature. Basic (2N NaOH aq. solution) workup and extraction with ether (2 × 50 ml) afforded 3.6 g of the terminal epoxide **8** (97% pure, yield 85%). HKR of 1.00 g of **8** (7.0 mmol) with Co(II) salen (Jacobsen's) catalyst **9** [prepared by 1 hr stirring of 20 mg of (*R*,*R*)-N,N′-bis(3,5-di-*tert*-butylsalicylidene)-1,2-cycloxehane-diaminocobalt with 0.15 ml of toluene and 0.035 ml of acetic acid, and subsequent solvent evaporation in vacuo] in 2 ml of dry THF and 0.80 eq. of H_2O (0.1 ml) was allowed to proceed at room temperature for 72 hr. (2*R*,7*R*)-1,2-Epoxy-7-hydroxyoctane (**10**) was separated from the catalyst and unwanted (2*S*,7*R*)-1,2,7-octanetriol (**11**) by flash chromatography [10 g of SiO_2; ether/hexane (1:1) as eluent] to yield the epoxyalcohol **10** (92% pure). Without any further purification, **10** was treated with excess of freshly prepared pentylmagnesium bromide (21 mmol) and catalytic amounts (0.40 g, 2.1 mmol) of CuI at −25°C for 1 hr. After warming to room temperature and usual workup, the (2*R*,7*S*)-stereoisomer of diol **12** (80% pure) was obtained. The crude mixture containing **12** was esterified with acetic anhydride (2 ml) and pyridine (5 ml) for

TABLE 1. STEREOISOMERIC COMPOSITON OF THE FOUR INDIVIDUAL STEREOISOMERS OF 2,7–DIACETOXYTRIDECANE (**4**)

	Stereoisomeric composition (%)			
Stereoisomer	*RR*-**4**	*RS*-**4**	*SR*-**4**	*SS*-**4**
RR-**4** (98.6 ee)	99.3	Nd	0.7	Nd
RS-**4** (99.0 ee)	Nd	99.5	0.1	0.4
SR-**4** (85.8 ee)	Nd	Nd	92.9	7.1
SS-**4** (94.6 ee)	Nd	Nd	2.7	97.3

Note. ee = enantiomeric excess, Nd = Not detectable.

12 hr at room temperature. Workup and column purification yielded *RS*-**4** (0.90 g, 2.91 mmol, 35% overall yield, 97% chemically pure; for stereoisomeric purity see Table 1).

Behavioral Experiments

Responses of male *A. aphidimyza* to conspecific females or synthetic candidate pheromones were tested in the laboratory in Y-shaped Pyrex glass olfactometers (stem: 20 cm × 2.5 cm ID; side arms: 18 cm at 120°) (Takács et al., 1997) and in experimental greenhouse compartments (3.5 × 12.2 × 3.5 m gutter; Pacific Agri-Food Canada Research Centre, Agassiz, British Columbia).

Olfactometer experiments in the laboratory were conducted under red light during the insects' scotophase. Five virgin female *A. aphidimyza* enclosed in wire mesh, or 1 ng of candidate pheromone dissolved in pentane, served as treatment stimuli, whereas empty wire mesh or pentane at equivalent quantity served as control stimuli. Synthetic pheromone or solvent was micropipetted onto Whatman #1 filter paper (1 cm diam.) placed near (5 cm) the orifice of each side arm of the Y-tube. For each replicate, a new (soapy water-cleaned and oven-dried) Y-tube, insect, and filter paper were used, with test stimuli randomly assigned to side arms. Charcoal-filtered air, drawn through the apparatus at 1 l/min with a water aspirator, carried volatiles from odor sources through the stem of the olfactometer. Thirty seconds after placement of stimuli, a single 1–3-d-old male *A. aphidimyza* was released from a holding tube into the stem of the olfactometer. Those insects that moved within 20 min >10 cm into a side arm were classed as responders; all others were classed as nonresponders and were not included in statistical analyses. Olfactometer experiments tested attractiveness of (a) live female *A. aphidimyza* (Exp. 1); (b) individual stereoisomers of 2,7-diacetoxytridecane (**4**) (*RS*-, *RR*-, *SR*-, or *SS*-**4**) (Exp. 2–5); and (c) stereoisomeric **4** (Exp. 6).

Two greenhouse compartments stocked with pepper plants, *C. annuum* 'Enza 444' (20–30 cm tall), were used to test the response of male *A. aphidimyza* in experiments 7 and 8 to sticky traps (Gray et al., 1984) baited with candidate pheromones released from filter paper dispensers (see above). Two experimental replicates separated by 5 m were run concurrently in each of the two compartments. For each replicate, six traps were suspended 30 cm above ground in a circle with 1 m spacing between traps. Five hundred *A. aphidimyza* pupae obtained from Applied Bionomics just prior to eclosion of adults were placed in the centre of the circle of traps in the opened shipping container. Seven days later, male *A. aphidimyza* captured in traps were recorded. Experiment 7 tested each of the four stereoisomers of **4** singly and in quaternary combination, and experiment 8 tested whether nonpheromonal stereoisomers, when added to the pheromone, reduced its attractiveness.

Data from olfactometer experiments 1–6 were analyzed by chi-square tests. Data of greenhouse experiments 7 and 8 were analyzed by nonparametric analyses of variance (Wilcoxon/Kruskal–Wallis Test) followed by comparison of means by Tukey–Kramer HSD test with JMPIN version 3.2 (SAS, 1997). In all analyses, $\alpha = 0.01$.

RESULTS AND DISCUSSION

GC-EAD analyses of pheromone gland extract of female *A. aphidimyza* revealed a single candidate pheromone (CP in Figure 2) that elicited responses from male antennae. Retention indices (RI) (van den Dool and Kratz, 1963) of CP [RI (relative to alkane standards): 1889 (DB-5), 2422 (DB-210), 2487 (DB-23)] and RI intercolumn differentials suggested that CP was a di-acetate. CP had the same RIs and antennal stimulatory activity in GC-EAD analyses of hydrogenated pheromone extract, further suggesting that it was a saturated molecule.

Considering that most of the presently identified cecidomyiid pheromones consist of a 13-carbon molecular chain with (at least) one acetate group in C2 (Foster et al., 1991; Harris and Foster, 1991; Millar et al., 1991; Hillbur et al., 1999,

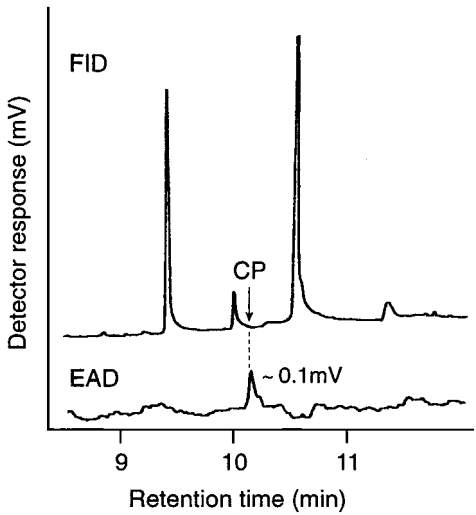

FIG. 2. Flame ionization detector (FID) and electroantennographic detector (EAD: male *Aphidoletes aphidimyza* antenna) responses to an aliquot of 1 female equivalent of pheromone gland extract of female *A. aphidimyza*. Chromatography: Hewlett Packard 5890A equipped with a DB-5 column; linear flow velocity of carrier gas: 35 cm/sec; injector and FID detector 240°C; temperature program: 50°C (1 min), 20°C per min to 280°C. CP = candidate pheromone.

2000, 2001; Gries et al., 2002), the molecular structure of CP was approximated by synthesizing various diacetoxytridecanes, with one acetoxy conservatively kept in C2 and the second acetoxy placed in C6, C7, C8, C9, C10, C11, or C12. Synthetic 2,7-diacetoxytridecane (compound **4** in Scheme 1), cochromatographing with CP on all analytical columns and eliciting comparable antennal responses, proved to be the candidate pheromone to be synthesized stereospecifically (Figure 1), and to be tested for attraction of male *A. aphidimyza*.

In Y-tube olfactometer experiments, caged virgin female *A. aphidimyza* (Exp. 1) and *RS*-**4** (Exp. 2) were the only test stimuli that significantly attracted males (Figure 3), clearly revealing the absolute configuration (2*R*,7*S*) of the *A. aphidimyza* pheromone. Failure of stereoisomeric **4** (containing all four

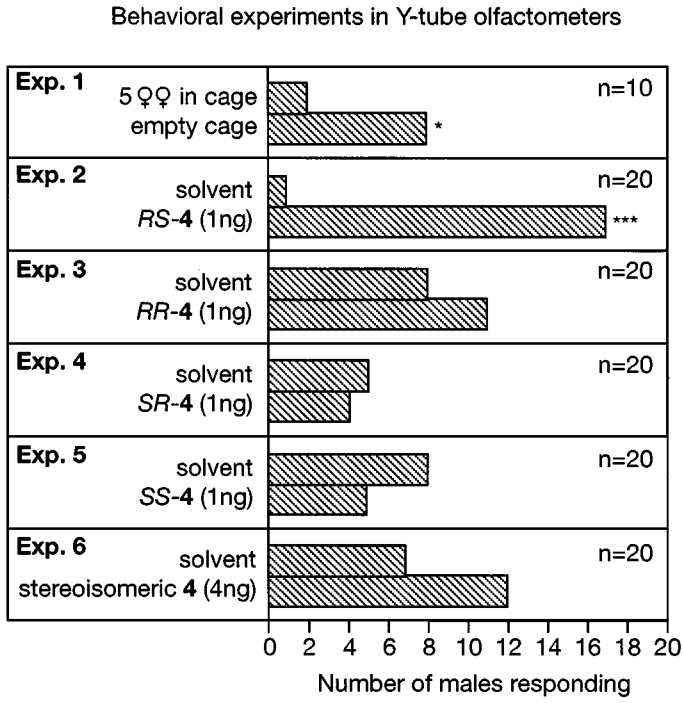

FIG. 3. Response of male *Aphidoletes aphidimyza* in Y-tube olfactometer experiments 1–6 to live virgin female *A. aphidimyza* or synthetic candidate pheromones. Note: (1) Compound abbreviations: *RS*-**4** = (2*R*,7*S*)-diacetoxytridecane; *SR*-**4** = (2*S*,7*R*)-diacetoxytridecane; *RR*-**4** = (2*R*,7*R*)-diacetoxytridecane; *SS*-**4** = (2*S*,7*S*)-diacetoxytridecane; (2) The volume of pentane (1 μl) as a solvent in treatment and control stimuli was identical; (3) Bars with asterisks indicate a significant preference for a particular treatment (chi-square test, $^{*}P < 0.05$, $^{***}P < 0.001$).

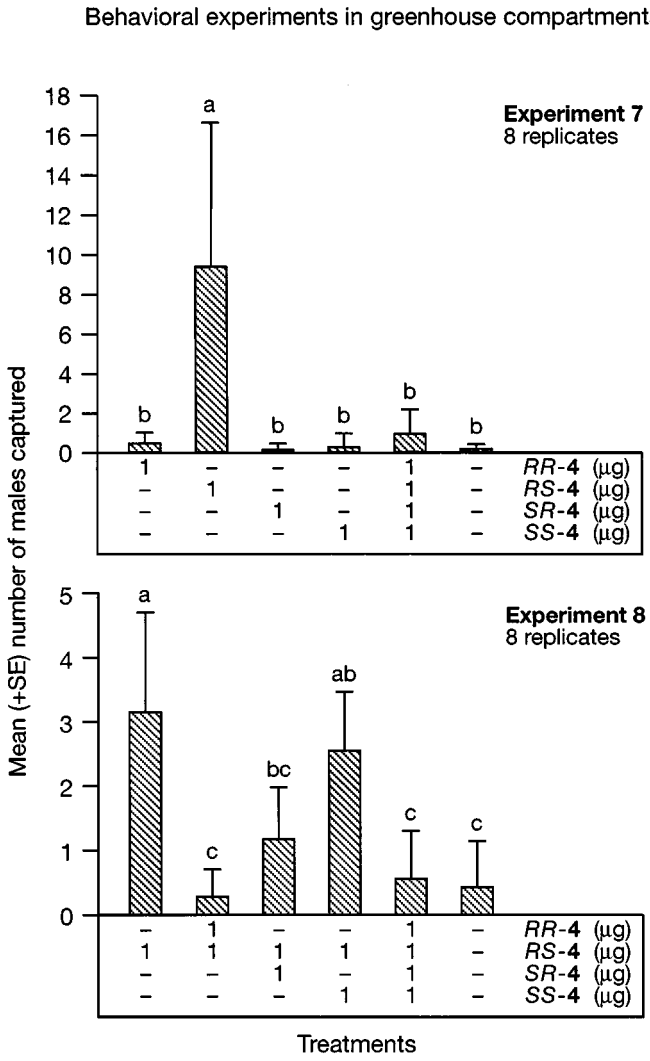

FIG. 4. Mean (+SE) number of male *Aphidoletes aphidimyza* captured in sticky traps baited with individual stereoisomers (*RR*-, *RS*-, *SR*-, or *SS*-**4**), or a stereoisomeric mixture, of 2,7-diacetoxytridecane (**4**) (Exp. 7), or baited with the pheromone (2*R*,7*S*)-diacetoxytridecane (*RS*-**4**) alone and in combination with either one or all of (2*R*,7*R*)-, (2*S*,7*R*)-, and (2*S*,7*S*)-diacetoxytridecane (*RR*-, *SR*-, and *SS*-**4**) (Exp. 8). Bars in each experiment with the same letter superscripts are not significantly different; nonparametric analyses of variance (Wilcoxon/Kruskal–Wallis Test) followed by comparison of means by Tukey–Kramer HSD test.

stereoisomers including the pheromone) in experiment 6 to attract male *A. aphidimyza* was attributed to inhibitory characteristics of one or more nonpheromonal stereoisomers.

Trapping experiments in greenhouse compartments (Figure 4) confirmed the absolute configuration (2*R*,7*S*) of the pheromone, and revealed inhibitory characteristics of *RR*-**4** and *SR*-**4**. In their presence, the attractiveness of the pheromone was reduced to levels not significantly different from those of unbaited control traps. Analogous results were obtained in bioassay experiments with male pea midges, *Contarinia pisi* (Hillbur et al., 2001). Response of male *C. pisi* to the pheromone blend was strongly inhibited by the presence (20%) of nonpheromonal stereoisomers.

(2*R*,7*S*)-Diacetoxytridecane, the pheromone of zoophagous *A. aphidimyza*, resembles the pheromones of phytophagous cecidomyiids [(*E*)-10-tridecen-(*S*)-2-yl acetate: Hessian fly, *Mayetiola destructor* (Foster et al., 1991; Harris and Foster, 1991; Millar et al., 1991); (2*S*,11*S*)-diacetoxytridecane, (2*S*,12*S*)-diacetoxytridecane, and 2-acetoxytridecane: *C. pisi* (Hillbur et al., 1999, 2000, 2001); (2*S*,7*S*)-nonanediyl dibutyrate: orange wheat blossom midge, *Sitodiplosis mosellana* (Gries et al., 2000); and (*Z*,*Z*)-4,7-tridecadien-(*S*)-2-yl acetate: Douglas-fir cone gall midge, *Contarinia oregonensis* (Gries et al., 2002)]. Despite their contrasting feeding ecology, these midges appear to have a common, diet-independent pathway for pheromone biosynthesis. Investigating these pathways, however, will remain challenging, as pheromone titers in most species are very low.

Aphidoletes aphidimyza are relatively expensive biological control agents (Gilkeson, 1987; van Lenteren et al., 1997). Lures with synthetic pheromone of *A. aphidimyza* could be developed to detect the presence of, or assess population densities of *A. aphidimyza* in commercial greenhouses. Ultimately, such information may help growers decide when release of these costly agents for aphid control is warranted.

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GROWTH INHIBITION AND ROOT ULTRASTRUCTURE OF CUCUMBER SEEDLINGS EXPOSED TO ALLELOCHEMICALS FROM RYE (*Secale cereale*)

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Abstract—Inhibition of "Calypso" cucumber seedling growth by rye allelochemicals, 2(3*H*)-benzoxazolinone BOA and 2,4-dihydroxy-1,4(2*H*)-benzoxazin-3-one DIBOA, was studied by analyzing the growth of seedling tissues and organs. Light and electron microscopy of seedling root cells were also carried out to investigate the mechanism(s) of root growth inhibition and mode of action of these compounds. BOA inhibited root elongation and reduced the number of cucumber lateral roots by 77 and 100% at 0.1 and 0.43 mg BOA/ml deionized (DI) water, respectively. DIBOA also inhibited root growth, but did not affect the number of lateral roots. BOA increased size of cucumber cortical root cells fivefold, but DIBOA had no effect. Both compounds reduced the regeneration of root cap cells and increased the width of cortical cells resulting in increased root diameter. BOA and DIBOA caused increased cytoplasmic vacuolation, reduced ribosome density and dictyosomes, reduced number of mitochondria, and reduced lipid catabolism. Starch granules in amyloplasts of seedling roots treated with BOA and DIBOA were also greatly reduced compared to the control. Changes in cellular ultrastructure indicated that BOA and DIBOA reduced root growth by disrupting lipid metabolism, reducing protein synthesis, and reducing transport or secretory capabilities.

Key Words—Allelopathy, BOA, DIBOA, root cell ultrastructure.

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INTRODUCTION

Natural compounds of high allelopathic activity such as those produced by rye, *Secale cereale* L., are good resources for novel herbicide chemicals. Advancement in research for natural herbicides depends greatly on understanding the mechanism of action of these compounds in plants and their behavior in soil. The efficacy of *S. cereale* residues in suppressing weeds has been documented in field studies (Barnes and Putnam, 1983; Shilling et al., 1985; Weston, 1990; Burgos and Talbert, 1996). The weed suppressive ability of *S. cereale* is attributed to two major compounds 2,4-dihydroxy-1,4(2*H*)-benzoxazin-3-one (DIBOA) and its degradation product 2(3*H*)-benzoxazolinone (BOA) (Barnes et al., 1987). These compounds are collectively called benzoxazolinones or benzoxazinones. In general, small-seeded species e.g., lettuce, *Lactuca sativa* L., and Palmer amaranth, *Amaranthus palmeri* S. Wats., are more sensitive to these compounds than large-seed species e.g., pitted morningglory, *Ipomoea lacunosa* L., and hemp sesbania, *Sesbania exaltata* L. (Burgos and Talbert, 2000). However, there is a wide range of differential tolerance to these compounds within a seed size class. For instance, among large-seeded crops, sweet corn, *Zea mays* var. *rogusa*, showed the most tolerance to aqueous extracts from *S. cereale* shoots (Burgos and Talbert, 2000). Similarly, among small-seeded weed species, large crabgrass, *Digitaria sanguinalis* L. Scop., showed the most tolerance to aqueous extracts of *S. cereale*. Seed size alone does not explain the tolerance of some species to these compounds. As in commercial herbicides, differential sensitivity of the target site or differential capability of a species to detoxify the compound could contribute to tolerance. Because of different levels of tolerance and the dose of toxic compounds at a given time, some large-seeded crops could be injured by residues of allelopathic cover crops. In petri dish bioassays, 200 ppm of either BOA or DIBOA reduced cucumber, *Cucumis sativus* L., root growth 44 and 69%, respectively (Chase et al., 1991). In another study, 0.37 and 1.05 mM of DIBOA and BOA, respectively, caused >50% root inhibition of cress, *Lepidium sativum* L."Curly," (Barnes et al., 1987). The persistence of these compounds in soil has been studied (Yenish et al., 1995). The mechanism of action of these compounds in plants is not well understood. Thus far, the strongest basis for the deleterious effects of benzoxazinones is their ability to inhibit energy transduction, thus, eventually reducing photosynthetic efficiency. It is reported that DIBOA and BOA inhibit the activity of plasma membrane H^+-ATPase from roots of oats, *Avena sativa* L. "Jumbo" (Friebe et al., 1997). Similarly, the DIBOA analogue (DIMBOA) from corn, *Zea mays* L., inhibits CF_1ATPase from chloroplasts of spinach, *Spinacea oleracea* L. (Queirolo et al., 1983), as well as electron transport in *Z. mays* (Massardo et al., 1994). Therefore, susceptible plants would appear chlorotic. Reduction of root and shoot growth of germinating seedlings has been attributed to inhibition of auxin-induced growth (Hoshi et al., 1994) as well as the enhancement of cell wall peroxidase activity, which leads to increased production

of H_2O_2 and premature lignification of cell walls (Gonzales and Rojas, 1999). This study was conducted to compare inhibitory effects of BOA and DIBOA on germination and seedling growth of *C. sativus* and to identify morphological and ultrastructural changes in *C. sativus* root cells resulting from BOA and DIBOA treatments.

METHODS AND MATERIALS

DIBOA Extraction. Shoot tissue from field-grown *S. cereale* "Maton" was harvested at booting stage (prior to panicle exertion). Tissue was oven-dried for 3 d at 60°C, cut to 20-cm portions, and ground to pass a 40-mesh screen in a Wiley mill (model 4). Extraction procedure for DIBOA was based on the method of Barnes et al. (1987) with minor modifications. Ground tissue, 30 g, was extracted with deionized (DI) water (1:10 (w/v)), homogenized in a Waring blender for 6 min and allowed to stand at room temperature for 20 min. The extract was filtered through six layers of cheesecloth, and 300 ml acetone were added to the aqueous extract. The solution was centrifuged (Beckman model J-6B) at 3046*g* for 10 min at 2°C. The filtrate was decanted and extracted into 400 ml diethyl ether ($\times 2$) in a separatory funnel. Ether fractions were combined, dried using anhydrous Mg_2SO_4, filtered, and the solvent was evaporated under vacuum in a rotary evaporator at 45°C. The residue was redissolved in 10 ml methanol, and the volume was reduced to 1.5 ml under a flow of N_2. The solution was applied to a 20 $\times$ 20 cm Whatman PLK5F Silica Gel 150 Åthin-layer chromatography (TLC) plate, 1000 μm, using 25-μl syringe in three applications. Samples were allowed to dry completely between applications. The chromatographic chamber was equilibrated for 30 min, and the TLC plate was developed in 9:1 $CHCl_3$:CH_3OH mobile phase. Spots were visualized with an ultraviolet (UV) lamp (254 nm). DIBOA was visualized by using a spray of 5% $FeCl_3$ in 95% ethanol, acidified to pH 2 (Barnes et al., 1987; Nair et al., 1990). DIBOA reacts blue to $FeCl_3$. The unsprayed portion of the DIBOA band was scraped, ground with a pestle in a porcelain mortar, placed in a test tube, and eluted into 25 ml of 2:1 $CHCl_3$:CH_3OH. The solution was vortexed (VWR Vortexer 2 G-560) for 10 min and centrifuged at 550*g* for 5 min, filtered through 0.42-μm Whatman disc filter, and reduced to about 0.75 ml. For purification of extracted DIBOA, the TLC procedure was repeated using Whatman LK6DF Silica Gel 60 Å 250 μm, and developed in 6:1 $CHCl_3$:CH_3OH mobile phase, where only one band was spotted. This band was scraped and eluted with 9 ml of 2:1 $CHCl_3$:CH_3OH. The solvent was evaporated, and the residue was crystallized in acetone/hexane yielding whitish crystals. The crystals were redissolved in methanol. The mass spectrum of DIBOA was verified by using gas chromatography–mass spectroscopy (GC) (Burgos et al., 1999).

BOA and DIBOA Bioassay. Six seeds of *C. sativus* "Calypso" were arranged in the middle of square culture dishes (8.5 × 8.5 × 1.5 cm) lined with Whatman no. 1 filter paper. Technical BOA (Sigma-Aldrich Chem. Co., St. Louis, MO.) was applied at 0.3, 0.6, 0.9, 1.2, and 1.5 mg/dish in a total volume of 3 ml DI water in three replications. Technical grade was the purest form (98%) of BOA available. It showed only one peak in the HPLC, and one band in the TLC procedures. There were no other inhibitory compounds in this formulation. Control treatments received only DI water. Culture dishes were sealed with parafilm and incubated in the growth chamber, completely randomized, for 4 d at 28°C. Culture dishes were incubated at an angle to promote geotropic growth of roots to facilitate measurement of root length. The experiment was conducted twice. Purified DIBOA extract from shoot tissue of *S. cereale* was applied at 0.1, 0.2, 0.3, and 0.4 mg per dish in 3 ml DI water in three replications. The experiment was conducted twice. The dosage ranges were based on preliminary bioassays in which at least 50% root inhibition was obtained, with DIBOA showing higher phytotoxicity. Germination, root and shoot lengths, and number of lateral roots were recorded. Data were analyzed using general linear models procedure (Statistical Analysis System [SAS], 1998).

Microscopy. Root tips (about 3 mm) of seedlings germinated in BOA (0.3 mg/ml) and DIBOA (0.1 mg/ml) treatments were rinsed in distilled water and fixed for 2 hr in a modified Karnovsky's fixative (Karnovsky, 1965) containing 2% glutaraldehyde and 2% paraformaldehyde in cacodylate buffer pH 7.0. Root tips were postfixed for 2 hr in 1% OsO_4, stained *en bloc* overnight in 0.5% aqueous uranyl acetate in the refrigerator, dehydrated in a 30–100% ethanol series followed by propylene oxide, and embedded in Spurr's low viscosity epoxy resin (Spurr, 1969). Thin sections (about 80 nm), were cut with a diamond knife mounted on an ultramicrotome (LKB 2088 Ultrotome® V), and picked up with etched copper grids. Copper grids were etched by successively dipping them in 0.1 N HCl, distilled water, and 97% EtOH. Copper grids containing thin sections were stained with 2% uranyl acetate followed by lead citrate (Mollenhauer, 1974). Specimens were viewed in a JEOL 100 EX electron microscope at 80 kV.

Thick sections (0.5–1 μm) were cut using glass knives mounted on an ultramicrotome (Sorvall Porter-blum MT2-B). Sections were picked up with a wire loop and floated onto droplets of 10% acetone (in distilled water) on a glass slide, which was precoated with Haupt's adhesive. The slide was dried on a warming tray at 50°C. For staining, the slide was placed on a hot plate at 100°C, flooded with staining solution for 2 min, rinsed with distilled water, and dried on a warming tray at 50°C. The staining solution contained 1% toluidine blue and 1% azure II in 1% borax prepared using distilled water (adapted from Alsop, 1974; Warmke and Lee, 1976). Specimens were viewed under a bright field microscope. Light microscope examinations were conducted to monitor the location of cells viewed under transmission electron microscope and to compare cell sizes and other

histological changes between treated and untreated cells. Lengths and widths of 35 cortical root cells between 0.5 and 1 mm from the root tip were measured from light micrographs. Data were analyzed by analysis of variance (ANOVA) procedure in SAS.

RESULTS

Effects of BOA and DIBOA on Cucumber Seedlings. Neither BOA nor DIBOA inhibited germination of *C. sativus* at the rates used (Figure 1). Shoot elongation of *C. sativus* was more inhibited by BOA than root elongation. I_{50} values for root and shoot elongation were 0.48 and 0.11 mg/ml, respectively, for BOA, and 0.06 and 0.053 mg/ml, respectively, for DIBOA. Besides inhibition of root and shoot elongation, BOA also reduced the number of lateral roots 35% at 0.1 mg/ml, and completely inhibited lateral root formation at 0.4 mg/ml (Figure 2). Longitudinal section of untreated cucumber root within 2.0 mm from the root tip showed well-defined epidermis, cortex, and stele (Figure 3A). Epidermal cells in untreated roots were narrower and longer than those of treated roots, which indicates rapid root elongation in untreated roots (Figure 3A). In contrast, roots treated with BOA had short, wide cells that resulted in large, but short roots (Figures 1 and 3B). DIBOA also increased root diameter, mainly due to increased number of columns of cortical cells (Figure 3C, Table 1). Furthermore, cortical cells in DIBOA-treated roots were wider than they are long. Cytoplasm content was apparently reduced in BOA-treated cells, as was reflected in lightly stained cells of BOA-treated roots. Root cap cells were revealed by their less dense cytoplasm compared to the apical root meristem. It was, however, difficult to pinpoint the boundary of root cap cells in BOA- and DIBOA-treated roots because there was minimal differential staining of cell layers in treated roots (Figure 3B and C). There were 8–10 layers of root cap cells in the untreated root versus 2–3 in BOA-treated roots (Figure 4A and B). DIBOA, on the other hand, altered the cellular morphology of apical root meristem and root cap so severely, compared to the control, that it was difficult to delineate cell types (Figure 4C). Electron micrographs of cross sections of untreated cucumber root tips showed that columella statocytes contain typical amyloplasts packed with starch granules, numerous mitochondria, and darkly staining lipid granules in the cytoplasm (Figure 5A). Nuclei in these cells were usually evenly spherical and contained prominent nucleoli (Figure 5A). Corresponding cells in BOA-treated roots showed fewer amyloplasts with less and smaller starch granules, invaginated plastids, irregularly shaped and lobed nuclei, higher amount of lipids, and an increased number of vacuoles of various sizes relative to the control (Figure 5B). DIBOA caused greater vacuolation and higher lipid content than BOA, while showing the same irregularly shaped nuclei (Figure 5C). Cytoplasm of most cells was filled with a greatly increased number of lipid droplets. Furthermore,

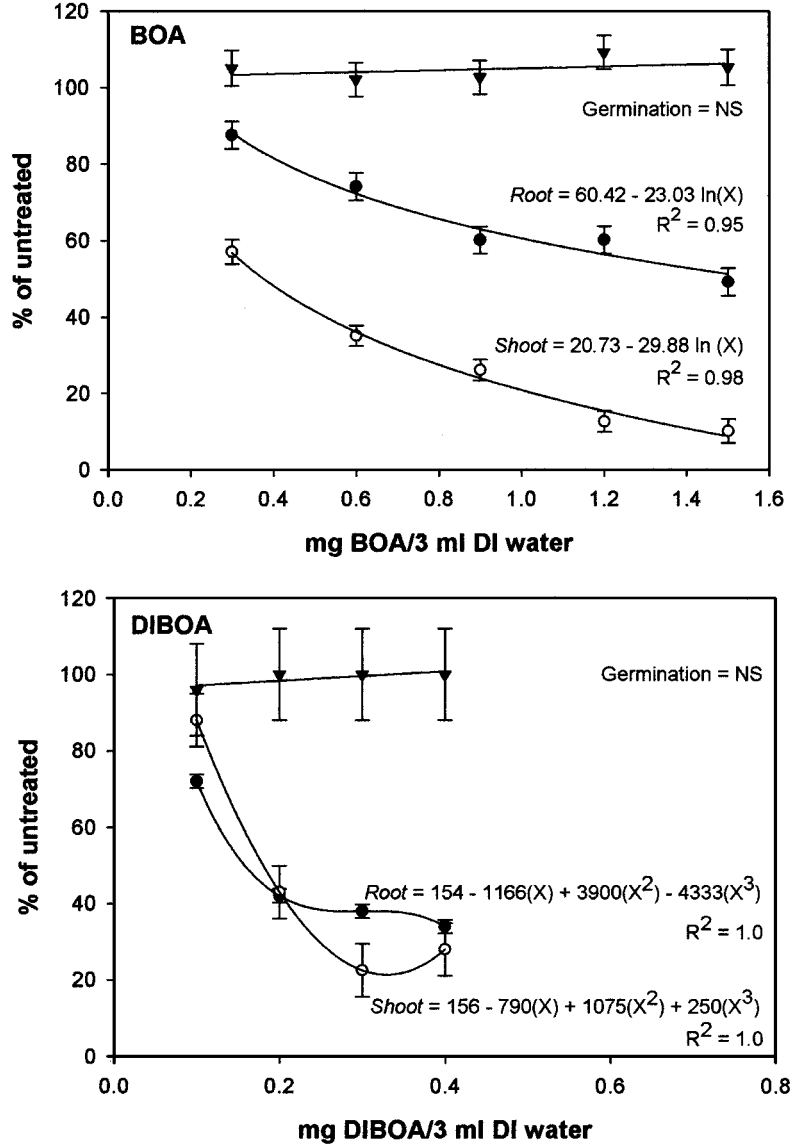

FIG. 1. Germination and root and shoot lengths (cm) of 4-day-old cucumber seedlings as affected by BOA and DIBOA in culture dish bioassays, dark-incubated at 28°C. Each data point is the average of 18 or more observations. Control (untreated) seedlings for the BOA treatments had an average root length of 3.71 cm, shoot length of 1.62 cm, and germination of 5.2 plants/dish; those in the DIBOA treatment had an average root length of 4.54 cm, shoot length of 2.68 cm, and germination of 5.7 plants/dish.

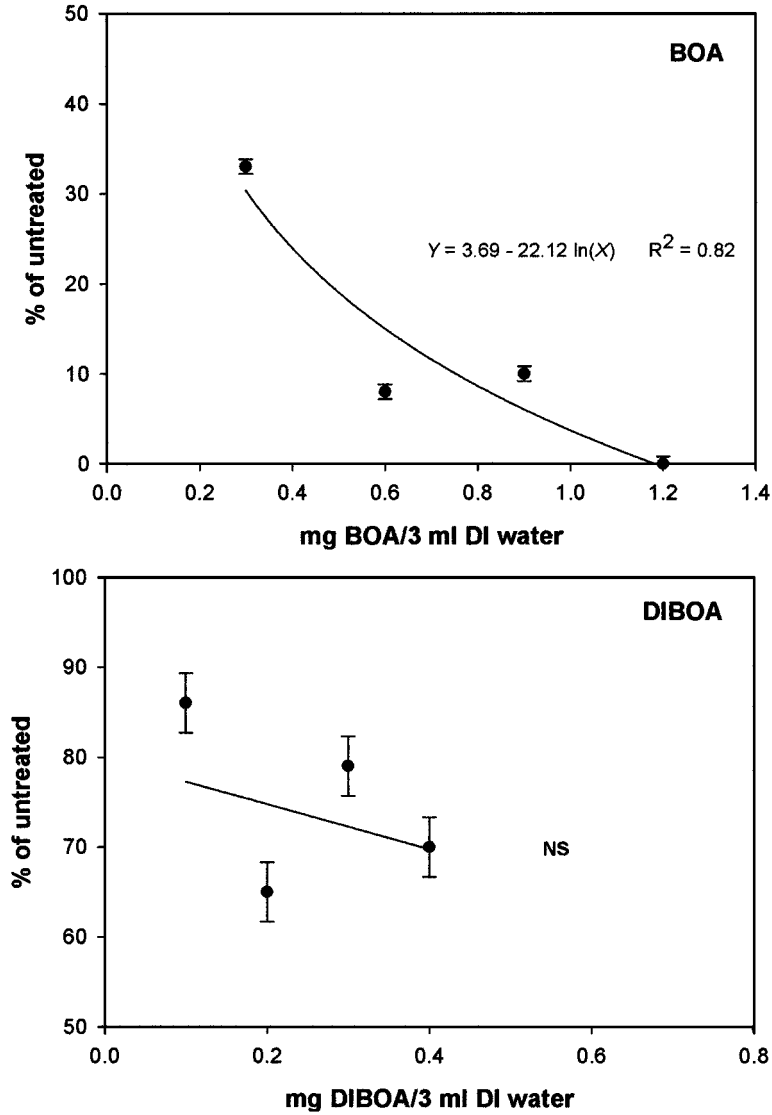

FIG. 2. Effects of BOA and DIBOA on the number of lateral roots per plant in 4-day-old cucumber seedlings in culture dish bioassays, dark-incubated at 28°C. Each data point is the average of 16 observations. Control (untreated) seedlings had an average 10.9 and 12.9 lateral roots/plant in the BOA and DIBOA treatments, respectively.

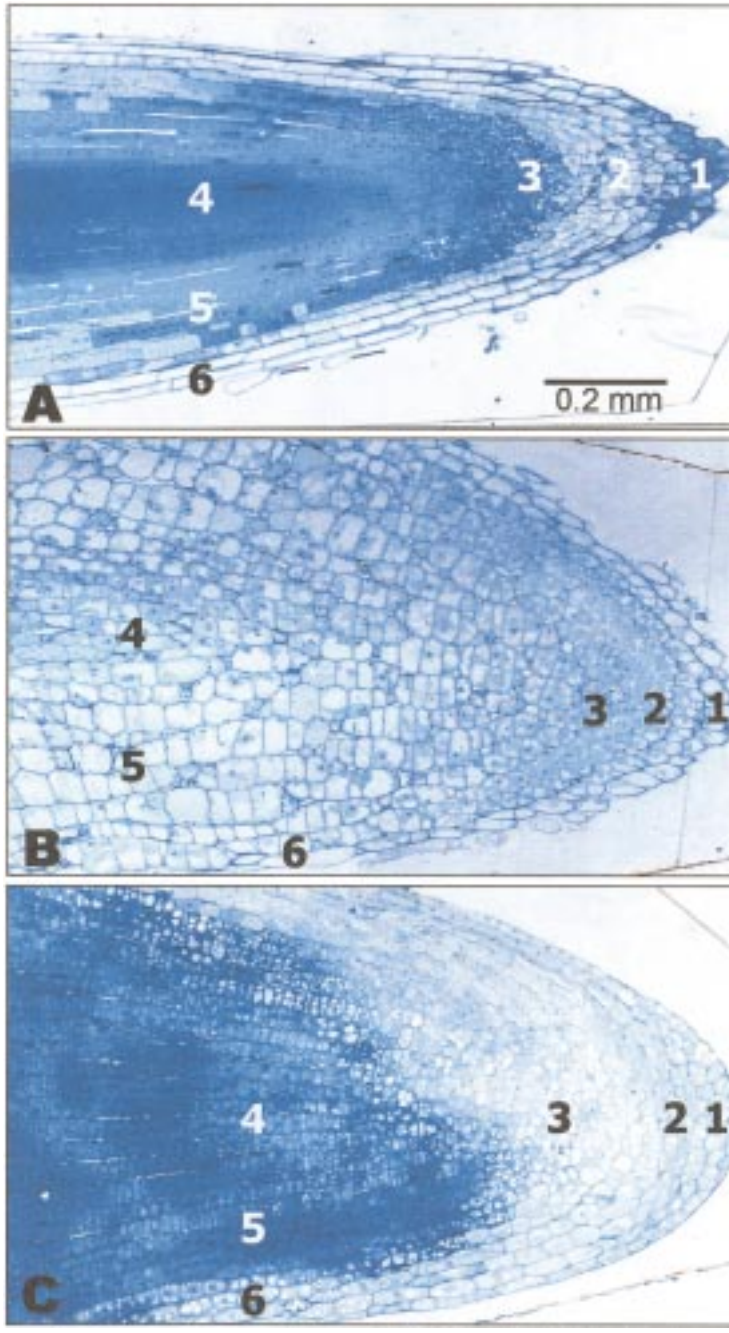
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TABLE 1. SIZE OF CORTICAL CELLS AND NUMBER OF CELL COLUMNS 0.5 TO 2.0 mm FROM THE TIP OF CUCUMBER ROOTS FROM 4-d-OLD SEEDLINGS AS AFFECTED BY BOA AND DIBOA

Treatment	Length (μm)[a]	Width (μm)[a]	Area (μm)[b]	No. of cell columns[b]
Untreated	29	49	1385	22
BOA	82	87	7126	22
DIBOA	28	64	1779	28
$LSD_{0.05}$	8	8	1109	3

[a] Measurements were taken from 35 cortical root cells between 0.5 and 1 mm from the root tip.
[b] Average of four replications. Each replication had five to six seedlings. A representative plant was taken from each replication for microscopic examinations.

lipid globules in DIBOA-treated cells were lightly stained compared to those of dark-staining lipids in BOA-treated cells.

Light micrographs revealed that cortical cells 0.5–1.0 mm above the root tip were greatly enlarged by BOA treatment compared to those of untreated cells (Figure 6A and B). The BOA treatment increased cell size approximately five times over the control (Table 1). DIBOA caused the cells to grow wider, but not longer, resulting in a tightly packed multiple columns of cells (Figure 6C). DIBOA had no significant effect on cell size, but increased cell width about 1.3 times over the control. DIBOA also increased the number of cell columns by 20%, but BOA had no effect. Electron micrographs of cucumber cortical root cells about 1 mm above the root tip showed that untreated roots have well-developed organelles, such as the golgi apparatus (dictyosomes), mitochondria, rough endoplasmic reticulum (RER), and highly abundant polyribosomes (Figure 7A). Vesicles budding off the maturation side of well-defined dictyosomes indicate active secretory function. BOA-treated cortical cells displayed bleached cytoplasm owing to significant loss of polyribosomes and ground substance in the cytoplasm (Figure 7B). Mitochondria appeared electron-dense, and dictyosomes were not well defined. There was

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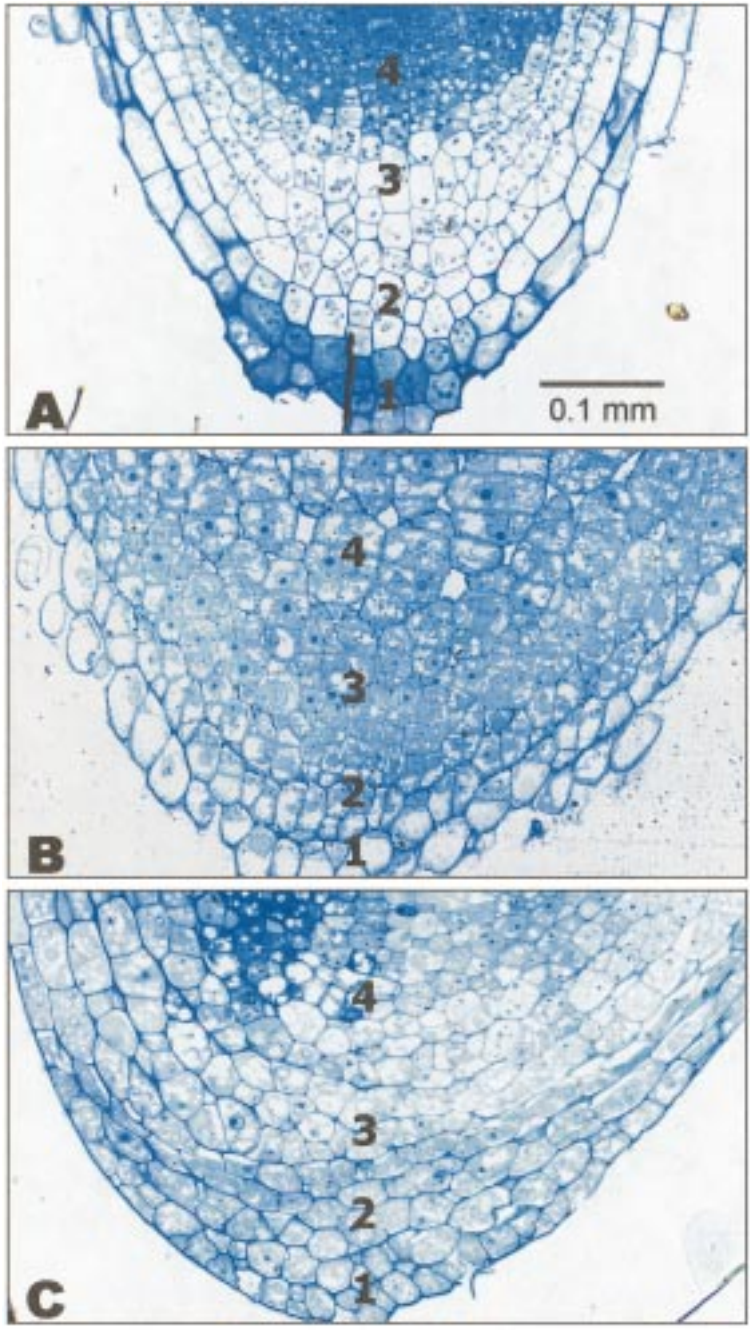
FIG. 3. Longitudinal section of cucumber root tips. A. Untreated root tip. Peripheral root cap cells are stained more densely than those of collumellar root cap cells due to the presence of mucilage (1). Columella root cap cells (2), which arose from the apical root meristem, are composed of calyptrogen, the meristematic layer of root cap cells and the columella statocytes, which contain amyloplasts for gravitational perception. The quiescent center (3) or apical root meristem is the origin of cortex, stele, epidermis, and root cap cells. Note the denser cytoplasm compared to the root cap and epidermal cells (6). Stele (4) and cortex (5) are also shown. B. BOA-treated root showing no differential staining between cell types, in contrast to untreated roots. Most cells, especially those of the cortex, are greatly enlarged. C. DIBOA-treated root also showing lack of distinction between apical root meristem and root cap cells.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less network of RER traversing the cytoplasm. DIBOA-treated roots had extremely large numbers of lipid droplets, numerous vesiculation, and a severely reduced density of polyribosomes (Figure 7C). Although there was a well-developed amyloplast in a DIBOA-treated cell, the overall number of amyloplasts was substantially reduced in BOA- and DIBOA-treated cells compared to the control (Figure 5).

DISCUSSION

Field-grown *S. cereale* "Maton" can produce 820 g/m^2 biomass, which contains 61 and 329 mg BOA and DIBOA, respectively (Burgos et al., 1999). Assuming that the field is irrigated with 2.54 cm-ha of water, and actual soil moisture content does not deviate much from this level during initial biomass decomposition, seeds of other species could potentially be exposed to about 11 mg/ml DIBOA and 2 mg/ml BOA. Actual amounts of each compound at any point in time could be less because of dynamic reactions (chemical, microbial, etc.), which contribute to degradation of these compounds in soil. Exudation of allelochemicals into the soil also occurs during the growing period of rye through the roots, thereby adding to the amount released from the decomposing shoot. The dosages used in the assay are at least 2.7 (DIBOA) and 50 (BOA) times greater than the theoretical amount released from shoot residue in the field. In the laboratory, it took 50 mg/ml DIBOA to reduce shoot growth of *C. sativa* 50%. Determination of I_{50} values for DIBOA and BOA in the field has not been done. Also, amounts of these compounds in soil have not been quantified in fields where *S. cereale* is grown or applied as mulch. In the field, total allelopathic activity of rye residue should be higher than in petri dish assays because of microbial activity. Soil microbes convert BOA into 2,2′-oxo-1,1′-azobenzene (AZOB), which is more toxic than DIBOA (Chase et al., 1991). The presence of these three toxic compounds in soil overlaps.

On the basis of I_{50} values, DIBOA was more inhibitory to root and shoot elongation of *C. sativus* seedlings than BOA in petri dish bioassays. Higher

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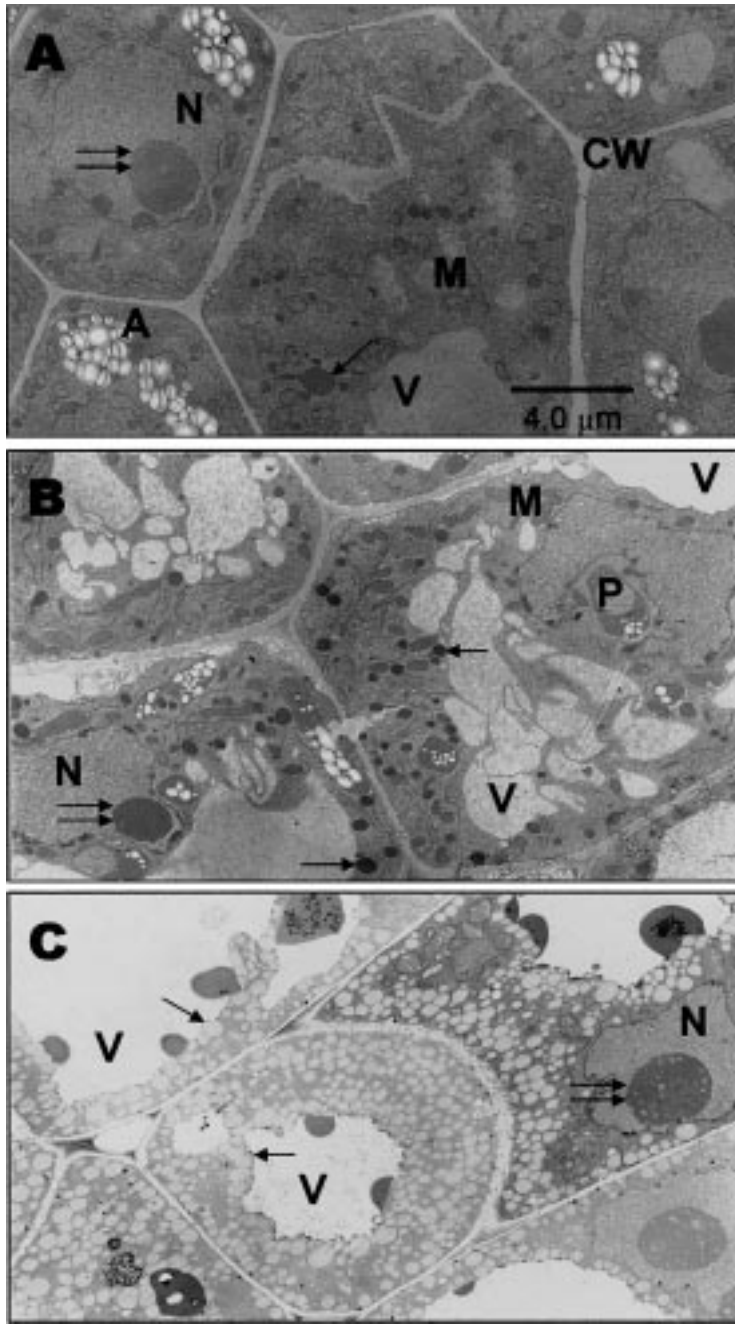
FIG. 4. Longitudinal section of cucumber root tips. A. Untreated. Shown are peripheral root cap cells (1), columella statocytes (2), and calyptrogen (3). In dicots, calyptrogen comprises two tiers of cells adjacent to the apical root meristem. Note that there are about eight tiers of root cap cells from the apical meristem to the outer peripheral cells. The quiescent center (4) has cells with the cuboidal shape of undifferentiated cells. B. BOA-treated root. Statocytes (1) and calyptrogen cells (2) are indistinct and there are fewer number of tiers. Also shown is the approximate location of apical root meristem (3) as indicated by the cuboidal shaped cells and cortical cells in the root apex that have not yet differentiated into stelar cells (4). C. DIBOA-treated root. Shown are peripheral root cap cells (1), columella root cap cells (2), the approximate location of apical root meristem (3), and cortical root cells in the root apex (4).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phytotoxicity of DIBOA compared to BOA has been documented in several plant species (Barnes and Putnam, 1987; Burgos and Talbert, 2000) and fungi (Wilkes et al., 1999). Although DIBOA has potent herbicidal activity, it is unstable and spontaneously degrades to the less potent BOA upon exudation (Barnes et al., 1987). Nevertheless, weed suppression is achieved, most likely because of the ensuing formation of AZOB, which contributes to overall phytotoxicity. Allelochemicals in rye residue placed on the soil surface are reduced 50% within 10–12 d after clipping (Yenish et al., 1995), but effective weed control lasts 4–6 wk depending upon weed species, weed density, and environmental conditions.

In petri dish assays, BOA inhibited the formation of lateral roots of *C. sativus*. Lateral root primordia arise from a group of cells in the pericycle, which become denser as they grow and start to divide in all directions (Mauseth, 1988). BOA apparently inhibited the differentiation of pericycle cells into lateral roots. DIBOA did not inhibit the formation of lateral roots, but the root cap cells of DIBOA-treated root were not as differentiated as those of the untreated root within the 1-mm region, indicating that DIBOA delayed cellular differentiation.

C. sativus has an open root meristem wherein there is no distinctive boundary between root cap cells and the apical root meristem. In dicots, meristematic root cap cells are usually the first two tiers of cells next to the apical root meristem (Barlow et al., 1984). BOA appeared to inhibit the differentiation of pericycle cells from the meristematic root cap cells, which eventually give rise to primordia of lateral roots, thereby inhibiting development of lateral roots. BOA reduced the differentiation of root cap cells, which paralleled the reduced development of lateral root primordia.

Increased cell width and reduced cell elongation, along with more cortical cell columns in the case of DIBOA teatment, resulted in reduced root elongation and increased root diameter. The reason for this unbalanced cell growth is uncertain, but this phenomenon was also observed in *C. sativus* in response to other allelochemicals. It could be a combined effect of the retardation of auxin-induced growth

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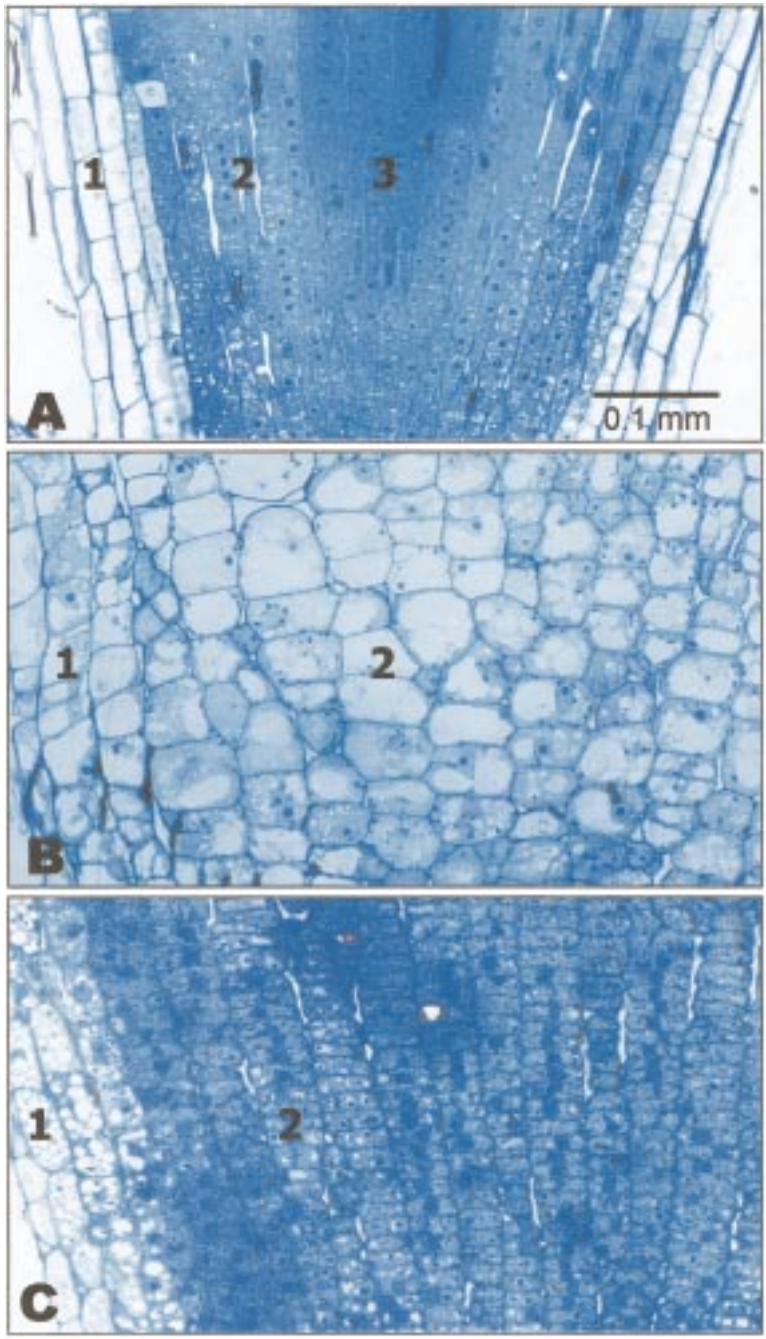
FIG. 5. Electron micrographs of cucumber root tip cells. A. Untreated root tip showing columella statocytes as indicated by the fully developed amyloplasts (A). The nucleolus (double arrows) and nucleus are prominent features of these cells. A few lipid globules (single arrow), several mitochondria (M), thin cell walls (CW), and few vacuoles (V) are also shown. B. BOA-treated root tip showing similar regions to those of the control has invaginated plastids (P) and amyloplasts that are not yet fully developed. Nuclei in these cells are not rounded, but lobulated. There is increased vacuolation (V) and lipid content (single arrows). Several mitochondria (M) are visible. C. DIBOA-treated root tip showing large vacuoles and a high amount of lipid droplets. No starch is seen in the plastids. Very few starch granules were located. Also, notice the light staining and more globular appearance of lipids (single arrows) compared to BOA treatment.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(Hoshi et al., 1994) and destabilization of cell walls due to enhanced activity of cell wall peroxidases (Gonzales and Rojas, 1999) in response to benzoxazolinone-type compounds. DIMBOA, a major allelochemical in *Z. mays*, has been shown to interfere with auxin-binding receptors, causing reduction in cell elongation (Hoshi et al., 1994). Because of stereochemical similarity, DIBOA might also do the same. Umbelliferone (7-hydroxycoumarin), an allelochemical commonly found in grasses, also decreased cell elongation and increased radial cell expansion in *C. sativus* roots (Jankay and Muller, 1976). Similarly, coumarin increased diameter of *C. sativus* roots above the apical meristem (Kupidlowska et al., 1994). Coumarin also inhibited cell elongation in the differentiation zone of radish, *Raphanus sativus* L., root tips (Aliotta et al., 1993).

Enlargement of vacuoles is necessary for growing cells to increase in size while maintaining turgor and shape (Mauseth, 1988). In mature cells, vacuoles often constitute 90% of the total protoplast volume (Mauseth, 1988). Normally, meristematic cells have microvacuoles in contrast to the large vacuoles observed in BOA- and DIBOA-treated root cells. Excessive vacuolation has also been observed in meristematic cells of white mustard, *Sinapis alba* L., treated with gramine and hordenine (Liu and Lovett, 1993). This is likely because vacuoles perform lytic functions in plant cells and help degrade toxins. Loss of ribosomes indicates reduced synthesis of proteins, that are intermediates for growth substances. Indirectly, reduced number of ribosomes leads to reduced growth. A related study showed that 6.8×10^{-4} M coumarin caused deterioration of cytoplasm, reduced ribosomes, inactivated the golgi apparatus, and degraded endomembranes in meristematic root cells of *C. sativus* (Kudiplowska et al., 1994). We also observed signs of inactivation of the golgi apparatus in this study, i.e., the extremely electron-dense dictyosome, indicating loss of structural integrity of the cisternal stack, and the absence of an endoplasmic reticulum network in close proximity to the dictyosome (Figure 7C). These, coupled with the overall scarcity of rough endoplasmic reticulum in the cytoplasm of BOA- and DIBOA-treated cells, indicate limited protein transport and reduced capability to export substances for cell development and growth.

One major difference between the effects of BOA and DIBOA was the higher amount of lipid globules in the DIBOA-treated root compared to BOA-treated ones.

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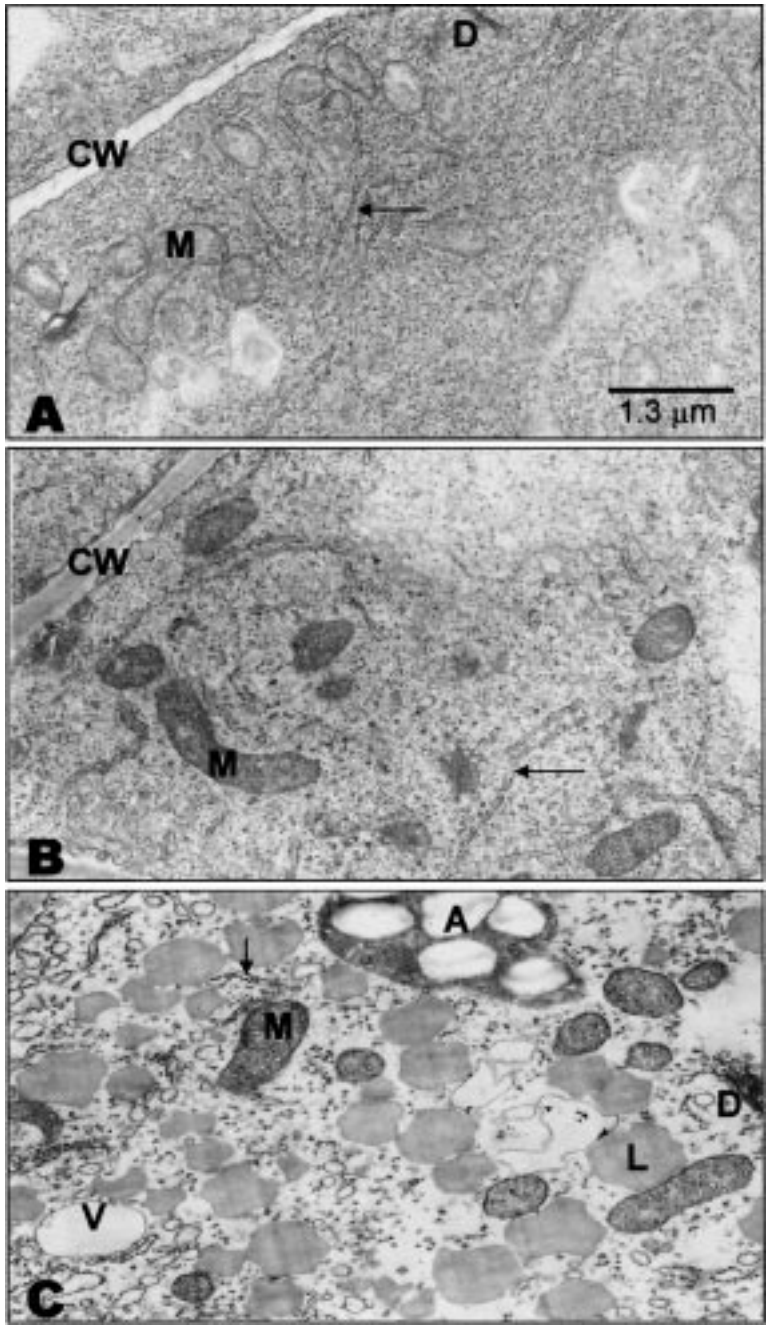
FIG. 6. Longitudinal sections (0.5–1 m) of cucumber root cells from the region immediately above the apical meristem. A. Untreated root tip showing epidermal cells (1) and cortex (2). Both sides of the root are visible because of the small root diameter. B. BOA-treated root showing epidermal cells (1) and cortex (2). Note the irregularity in cell shapes. Stele is not visible in this region under the same magnification as untreated root because cells and root diameter are large. C. DIBOA-treated root showing epidermal cells (1) and cortex (2). In this section, no stelar cells are visible, and cortical cells are short and wide, but regularly shaped in contrast to the BOA-treated root.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Lipid degradation increases during the germination process to sustain the energy requirements of growing seedling (Mauseth, 1988). Stored energy in lipids is released via the glyoxylate cycle in glyoxysomes of plant seedlings in coordination with the mitochondria. High amount of lipid globules in roots of 4-d-old seedlings suggests insufficient utilization of energy necessary for cells to grow and multiply. Lack of lipid degradation, in response to allelochemicals gramine and hordenine from barley, *Hordeum vulgare* L., has also been observed in root cells of *S. alba* (Liu and Lovett, 1993). Inhibition of lipid catabolism by DIBOA would result in reduction of ATP production, another cause for growth reduction or plant death in sensitive species. This suggests that DIBOA may have inhibited ATP production in the mitochondria. Various flavonoids found in some grasses and legumes have been reported to inhibit ATP production in mitochondria of *C. sativus* hypocotyls (Stenlid, 1970). BOA and DIBOA inhibit H^+-ATPase in the plasma membrane of roots of *A. sativa* and *Vicia faba* (Friebe et al., 1997), and BOA was less inhibitory to the enzyme than DIBOA. Also, related to perturbation of energy production in the plant, BOA and DIBOA had been reported as photosynthesis inhibitors, the tangible effect being reduction of chlorophyll production (Barnes and Putnam 1987). The analogue, DIMBOA, inhibits chloroplast ATPase (Queirolo et al., 1983) and electron transport both in the mitochondria and chloroplast (Massardo et al., 1994).

Ultrastructural changes in *C. sativus* root cells show that the action of BOA and DIBOA is multifaceted. There were indications of inhibition of protein synthesis, possible inhibition of enzyme systems in the mitochondrial membrane, and secretory malfunctions in different degrees caused by both allelochemicals. Which effect is primary or secondary is unclear. It is likely that these effects are interdependent and that these compounds have multiple modes of action. Allelochemicals act on more than one physiological process in the plant, hence, the difficulty in separating primary from secondary modes of action. For example, ferulic acid at 500 mM inhibited protein synthesis in *L. sativa* (Cameron and Julian, 1980); decreased stomatal conductance in sorghum, *Sorghum bicolor* (L.) Moench

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FIG. 7. Electron micrographs of longitudinal sections of cucumber roots about 1 mm from the root tip. A. Untreated cortical cells showing well-developed organelles such as rough endoplasmic reticulum (single arrow), dictyosomes (D), mitochondria (M), and abundant polyribosomes. The dictyosome shows vesicles budding off the maturation side, indicating active secretory function. B. BOA-treated cortical cells showing bleached cytoplasm due to the loss of polyribosomes, and ground substance in the cytoplasm. Mitochondria (M), and rough endoplasmic reticulum (arrow) are well contrasted due to the loss of polyribosomes and ground substance in the cytoplasm. C. DIBOA-treated cortical cells showing an extremely high number of lipid droplets (L), vacuole (V), mitochondria (M), dictyosome (D), amyloplast (A) and rough endoplasmic reticulum (arrow). Loss of polyribosomes is most severe.

(Einhellig et al., 1985); decreased chlorophyll content in soybean, *Glycine max* L. (Einhellig and Rasmussen, 1979); and decreased uptake of PO_4^{-3} (Glass, 1973) in *H. vulgare*, and K^+ (Harper and Balke, 1981) in oats, *Avena sativa* L. Low concentration of ferulic acid (250 mM) reduced the cell membrane potential in *H. vulgare* (Glass and Dunlop, 1974). In the field, the general effect of allelopathic activity of rye (or other species) includes reduced emergence, stunting, and chlorosis.

In summary, the overall effect of DIBOA on *C. sativus* was more pronounced than that of BOA. Both compounds increased root diameter, but reduced root length. BOA had larger, but fewer meristematic cells than controls. DIBOA had shorter cells, but had more columns of cortical cells than controls, resulting in increased root diameter. Both allelochemicals altered the ultrastructure of *C. sativus* roots. DIBOA has a greater potential herbicidal activity than BOA and, therefore, merits further investigations for practical applications in weed management. The rate of inhibition of ATP production at different levels of DIBOA needs to be verified using isolated mitochondria from sensitive plants.

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EARTHWORMS AND LITTER DISTRIBUTION AFFECT PLANT-DEFENSIVE CHEMISTRY

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Abstract—Studies on plant-defensive chemistry have mainly focused on plants in direct interaction with aboveground and occasionally belowground herbivores and pathogens. Here we investigate whether decomposers and the spatial distribution of organic residues in soil affect plant-defensive chemistry. Litter concentrated in a patch (vs. homogeneously mixed into the soil) led to an increase in the aucubin content in shoots of *Plantago lanceolata*. Earthworms increased total phytosterol content of shoots, but only when the litter was mixed homogeneously into the soil. The phytosterol content increased and aphid reproduction decreased with increasing N concentration of the shoots. This study documents for the first time that earthworms and the spatial distribution of litter may change plant-defensive chemistry against herbivores.

Key Words—Multitrophic interactions, decomposers, *Plantago lanceolata*, phytosterols, iridoid glycosides, organic residues, spatial heterogeneity.

INTRODUCTION

Few studies have addressed the potential effects of soil biota on plant-defensive chemistry (reviewed by Van Dam et al., 2003) and all of these studies deal with belowground organisms that directly interact with living plants (e.g., root herbivores). However, large decomposers, such as earthworms, that interact indirectly with plants via nutrients or soil structure have strong effects on plant growth and may also change plant chemistry (reviewed by Brussaard, 1999; Scheu, 2003). Furthermore, soil heterogeneity affects nutrient uptake, growth, and chemistry of

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plants (Robinson, 1994), since plant roots proliferate in nutrient-rich patches and "forage" for nutrients (Hutchings and de Kroon, 1994).

In a study investigating the effects of earthworms and litter distribution on plants of different functional groups (grass/forb/legume) and the reproduction of aphids (*Myzus persicae* Sulzer) (Wurst et al., 2003), we found that the presence of earthworms reduced aphid reproduction on *Plantago lanceolata* L. (Plantaginaceae). This was unexpected as other studies had suggested that earthworm activity increased aphid reproduction (Scheu et al., 1999; Wurst and Jones, 2003). We speculated that earthworms may have modified the defensive chemistry of *P. lanceolata* (Wurst et al., 2003) and decided to conduct further analyses of iridoid glycosides and phytosterols in *P. lanceolata* that are presented in this publication.

Iridoid glycosides (aucubin and catalpol) are important secondary metabolites in *P. lanceolata*. Jarzomski et al. (2000) documented that nutrient availability determined iridoid glycoside concentration to a greater extent than herbivory. Since earthworms and litter distribution affect nutrient availability to plants, it might also change the concentration of iridoid glycosides. Deterrent effects of iridoid glycosides on generalist insect herbivores are well documented (Bowers and Puttick, 1988; Puttick and Bowers, 1988).

Primary plant metabolites also affect herbivore performance (Karban and Baldwin, 1997) and may be important for plant defense. As well as being structural constituents of plant membranes, phytosterols are precursors of insect hormones and must be taken up with diet by insects that are unable to biosynthesize them directly (Svoboda et al., 1994). Since phytosterols are transported in the phloem sap (Lehrer et al., 2000), aphid performance may be affected by their concentration. Bodnaryk et al. (1997) reported reduced aphid reproduction on *Brassica napus* treated with a systemic fungicide that led to a depletion of sitosterol, campesterol, and stigmasterol. However, only one study has shown that aphids (*Schizaphis graminum*) can convert dietary phytosterols into cholesterol, a precursor to molting hormones, the ecdysteroids (Campell and Nes, 1983). Although many studies have suggested that aphids rely on their endocytobionts to synthesize sterols (reviewed by Ishikawa, 1989), there is strong evidence that endocytobiotic bacteria (*Buchnera* spec.) are not involved in the sterol nutrition of aphids (Douglas, 1998).

Phytosterol composition in leaves is affected by soil organisms such as VA-mycorrhiza (Dugassa-Gobena et al., 1996) and root-born endophytic fungi (Vidal and Dugassa-Gobena, 1999). In the latter study, phytosterols of tomato (*Lycopersicon esculentum*) and cabbage (*Brassica oleracea*) were affected by inoculation with endophytic fungi (*Acremonium* spec.) leading to changes in performance of diamondback moth (*Plutella xylostella*) on cabbage.

Here we investigate whether the presence of soil macrofauna (endogeic earthworms) and the spatial distribution of organic residues (homogeneous vs. patch) affect defensive chemistry (iridoid glycosides and phytosterols) of *P. lanceolata*.

METHODS AND MATERIALS

Analyses of iridoid glycoside and phytosterol contents were carried out with freeze-dried *Plantago lanceolata* L. (Plantaginaceae) plants from a greenhouse experiment. Since the plants were part of a more extensive study, further data on biomass, total N and ^{15}N content (mg) of the plants, and aphid reproduction were provided in a former publication (Wurst et al., 2003).

Greenhouse Studies. A total of 28 experimental containers (height 25 cm, diam. 10 cm) were set up in the greenhouse (16-hr light, night/day temperature 18/20˚C, humidity 60–70%). Half of the containers were filled with 600 g of a nutrient-poor mineral soil, and 1.43 g ^{15}N-labeled *Lolium perenne* L. (Poaceae) shoot litter (13.14 atom% ^{15}N) was placed as a "patch" in the middle of the container before a further 600 g of soil was added. The remaining 14 containers were filled with 1200 g of the same soil mixed homogeneously with 1.43 g ^{15}N-labeled *L. perenne* shoot litter.

Two individuals of *P. lanceolata* with approximately five leaves were transplanted from seedling trays into the containers. Four days later, two individuals of the endogeic earthworm *Aporrectodea caliginosa* (Savigny) (Lumbricidae) and one individual of *Octolasion tyrtaeum* (Savigny) (Lumbricidae) were placed into half of the containers of each litter distribution type ("earthworm treatment"). The experimental containers were watered with 50-ml distilled H_2O daily and redistributed randomly within the greenhouse every 2 weeks. In Week 3 of the experiment, one adult aphid of *Myzus persicae* (Sulzer) (Aphididae) was placed into a clip cage (height 2 cm, diam. 4 cm) on an intermediately aged leaf of each *P. lanceolata* individual. Seven days later, the number of offspring was counted. In Week 10, all plants were harvested. Plants were cut at ground level, freeze-dried, and weighed. The two individuals of *P. lanceolata* per pot were combined into a single sample.

Nitrogen. Freeze-dried shoot samples were ground into a powder and approximately 1 mg was weighed into tin capsules. Isotope ratio $^{15}N/^{14}N$ was measured by an elemental analyzer (NA 1500, Carlo Erba, Milan, Italy) coupled with a trapping box (type CN, Finnigan, Bremen, Germany) and a mass spectrometer (MAT 251, Finnigan, Bremen, Germany). Atmospheric nitrogen served as base for $\delta^{15}N$ calculation and acetanilide (C_8H_9NO, Merck, Darmstadt, Germany) as internal standard (Reineking et al., 1993).

Iridoid Glycosides. From each ground, freeze-dried shoot sample (three replicates per treatment; $N = 12$), 25 mg were extracted overnight in methanol (95%). The supernatant was filtered and discarded. Phenyl-β-D-glucopyranoside solution (200 μl; 2.5 mg/ml in 95% methanol) was added as internal standard, and the extract was evaporated to dryness. After partitioning, the ether layer was discarded and the water layer (that contains mainly iridoid glycosides and sugars) was evaporated to dryness. An aliquot was derivatized with Tri-Sil Z (Pierce Chemical

Company, Rockford, IL, USA) and analyzed by gas chromatography (Gardner and Stermitz, 1988; Stamp and Bowers, 2000).

Phytosterols. Each freeze-dried sample (500 mg; five replicates per treatment; $N = 20$) was dissolved with 20 ml of solvent (10 M KOH, 96% ethanol (1:5; v/v%) and 0.3% pyrogallol as antioxidant) in a water bath (80˚C) for 2.5 hr. Cholesterol solution (50 μl; 5 mg/ml in chloroform) was then added as an internal standard. Phytosterols were extracted by washing twice with 10-ml hexane and evaporating the hexane solution to dryness. After dissolving in 1.5-ml hexane, the extracts were transferred into autosampler vials and dried overnight in a thermo-block (50˚C). The residual was dissolved with 240 μl $N'-N'$-dimethylformamide, and 60 μl bistrimethylsilyltrifluoracetamide (BSTFA) were added for methylation (70˚C for 10 min). Samples were then injected into a gas chromatograph (modified after Newton, 1989; Dugassa-Gobena et al., 1996).

Statistical Analyses. Data were analyzed by analysis of variance (ANOVA) in a general linear model and regression analysis (Statistica, Statsoft 2001). The explanatory variables were litter distribution ("litter") and presence of earthworms ("earthworms"). The distribution of errors (Kolmogorov–Smirnov one-sample test) and the homogeneity of variances (Levene's test) were tested. The data were log-transformed if appropriate.

RESULTS

Iridoid Glycosides. Iridoid glycoside (aucubin and catalpol) content in shoots of *P. lanceolata* increased when the litter was concentrated in a patch ($F_{[1,9]} = 10.93$; $P = 0.016$). This was due to an increase of aucubin by 54% ($F_{[1,9]} = 15.02$; $P = 0.008$; Figure 1), as catalpol was not affected by the litter distribution ($F_{[1,9]} = 0.89$; $P = 0.383$; Figure 1). Earthworm presence did not affect iridoid glycoside content in shoots ($F_{[1,9]} = 1.44$; $P = 0.276$).

Phytosterols. When the litter was mixed homogeneously into the soil, total phytosterol, sitosterol, and campesterol content in shoots increased in the presence of earthworms by 75% ($F_{[1,18]} = 14.38$; $P = 0.002$), 81% ($F_{[1,18]} = 15.76$; $P = 0.001$), and 63% ($F_{[1,18]} = 12.33$; $P = 0.003$), respectively (Figure 2). No differences were observed when the litter was concentrated into a patch. Stigmasterol was not affected by earthworm presence ($F_{[1,18]} = 1.13$; $P = 0.305$) or litter distribution ($F_{[1,18]} = 0.01$; $P = 0.906$; Figure 2).

Nitrogen. Earthworms increased shoot N concentration (% w/w) by 112% when the litter was mixed homogeneously into the soil, but only by 40% in treatments with a litter patch ($F_{[1,27]} = 7.96$; $P = 0.009$ for the interaction litter $\times$ earthworms; Figure 3).

Iridoid Glycosides, Phytosterols, and Nitrogen. Iridoid glycosides were not correlated with shoot N concentration ($F_{[1,8]} = 0.14$; $P = 0.723$; $R^2 = 0.13$ for

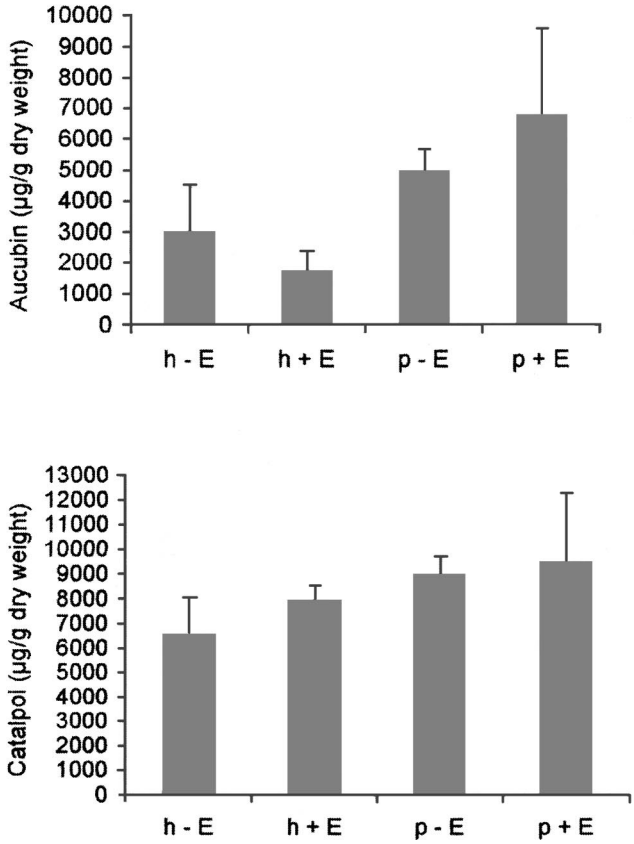

FIG. 1. Effect of litter distribution and earthworms [homogeneous without earthworms (h − E), homogeneous with earthworms (h + E), patch without earthworms (p − E), patch with earthworms (p + E)] on the aucubin and the catalpol content (μg/g dry weight) of *Plantago lanceolata* shoots (means + SD).

aucubin; $F_{[1,8]} = 0.46$; $P = 0.513$; $R^2 = 0.06$ for catalpol). Total phytosterol, sitosterol, and campesterol content increased with the concentration of N in shoots ($F_{[1,17]} = 33.97$; $P < 0.001$; $R^2 = 0.67$ for total phytosterols; $F_{[1,17]} = 32.83$; $P < 0.001$; $R^2 = 0.66$ for sitosterol; $F_{[1,17]} = 20.60$; $P < 0.001$; $R^2 = 0.55$ for campesterol; Figure 4). Stigmasterol was not correlated with the N concentration in shoots ($F_{[1,17]} = 1.93$; $P = 0.183$; $R^2 = 0.10$).

Correlations between Aphid Reproduction and Plant Chemistry. The number of offspring produced by *M. persicae* decreased with increasing shoot N concentration ($F_{[1,26]} = 7.68$; $P = 0.010$; $R^2 = 0.23$; Figure 5), but was not correlated with

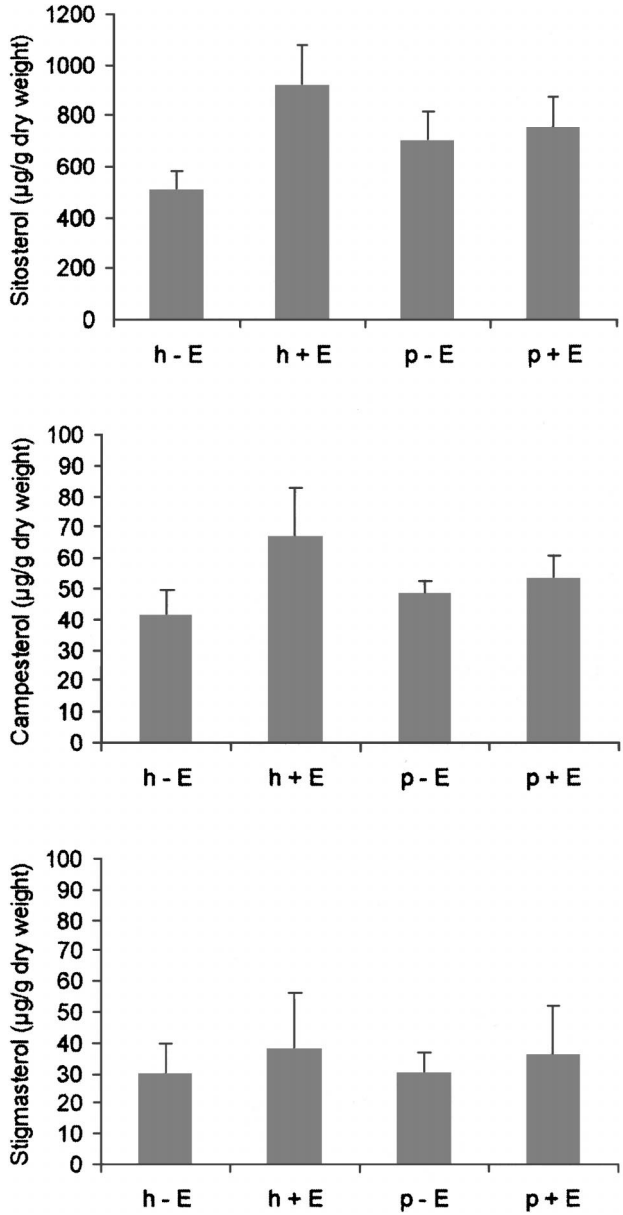

FIG. 2. Effect of litter distribution and earthworms [homogeneous without earthworms (h − E), homogeneous with earthworms (h + E), patch without earthworms (p − E), patch with earthworms (p + E)] on the sitosterol, campesterol, and stigmasterol content (μg/g dry weight) of *Plantago lanceolata* shoots (means + SD).

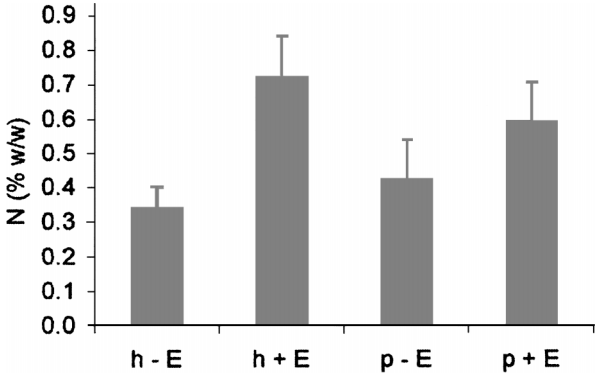

FIG. 3. Effect of litter distribution and earthworms [homogeneous without earthworms (h − E), homogeneous with earthworms (h + E), patch without earthworms (p − E), patch with earthworms (p + E)] on the nitrogen concentration (% w/w) of *Plantago lanceolata* shoots (means + SD).

the contents of aucubin ($F_{[1,8]} = 0.31$; $P = 0.596$; $R^2 = 0.04$), catalpol ($F_{[1,8]} = 3.14$; $P = 0.114$; $R^2 = 0.282$), sitosterol ($F_{[1,17]} = 0.30$; $P = 0.590$; $R^2 = 0.02$), campesterol ($F_{[1,17]} = 0.01$; $P = 0.938$; $R^2 < 0.001$), or stigmasterol ($F_{[1,17]} = 2.18$; $P = 0.158$; $R^2 = 0.11$).

DISCUSSION

Earthworms and the spatial distribution of organic residues significantly affected the plant chemistry of *P. lanceolata*. Aucubin content of shoots increased in the presence of a litter patch. Earlier studies documented that the aucubin concentration of *P. lanceolata* decreased with increasing fertilization, i.e., plant N concentration (Fajer et al., 1992; Jarzomski et al., 2000). Jarzomski et al. (2000) assumed that the iridoid glycoside concentrations reflected a physiological response to nutrient availability. In the present study, plants were not fertilized, and shoot N concentration and aucubin content were not correlated. The presence of a litter patch led to increased aucubin concentrations without influencing the N concentration of shoots. The mechanisms by which the spatial distribution of organic residues in soil affects plant secondary compounds are unknown and need further investigation.

The changed aucubin content did not affect reproduction of *M. persicae*. Gange and West (1994) reported increased levels of aucubin and catalpol in *P. lanceolata* in symbiosis with arbuscular mycorrhizal fungi (AMF), and improved growth and reproduction of *M. persicae* on the mycorrhizal plants. However, they

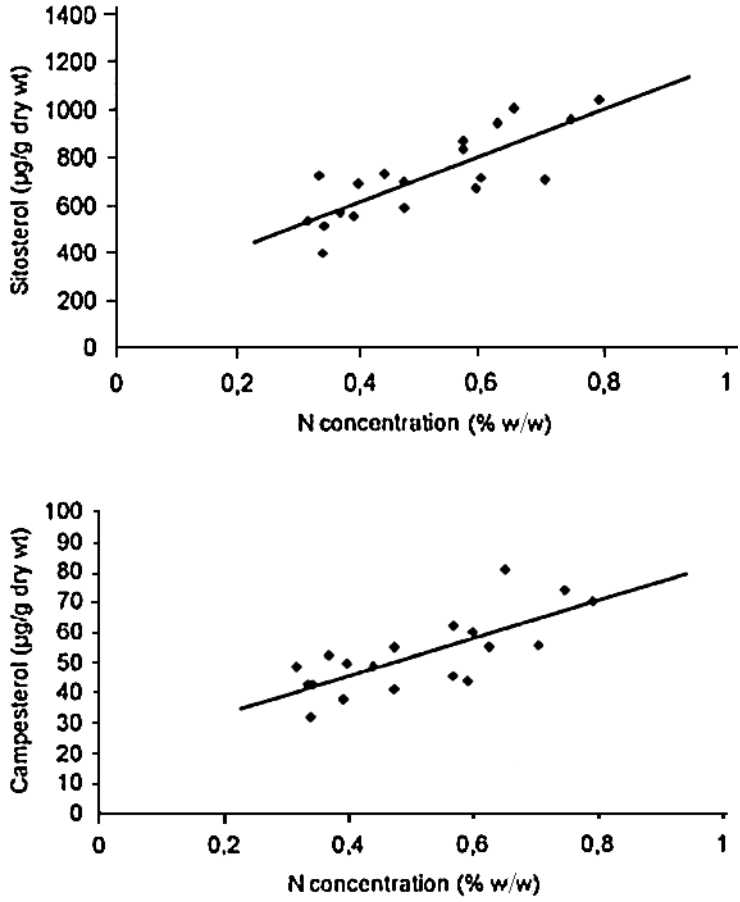

FIG. 4. Regression between N concentration (% w/w) and sitosterol content, and campesterol content (μg/g dry weight) in shoots of *Plantago lanceolata*.

suggested that plant morphology rather than chemistry was responsible for improved aphid performance on mycorrhizal plants. Increased levels of aucubin and catalpol in mycorrhizal plants presumably reduced growth, food consumption, and frass production of the generalist *Arctia caja* (Gange and West, 1994). Consistent with these findings, iridoid glycosides are known to deter generalist insect herbivores (Bowers and Puttick, 1988; Puttick and Bowers, 1988). Variation in iridoid glycoside content due to soil conditions (e.g., litter distribution, presence of AMF, fertilization) may influence aboveground plant–herbivore interactions. Darrow and Bowers (1999) documented higher levels of aucubin in *P. lanceolata* roots grown under nutrient limitation. However, nutrient availability did not change

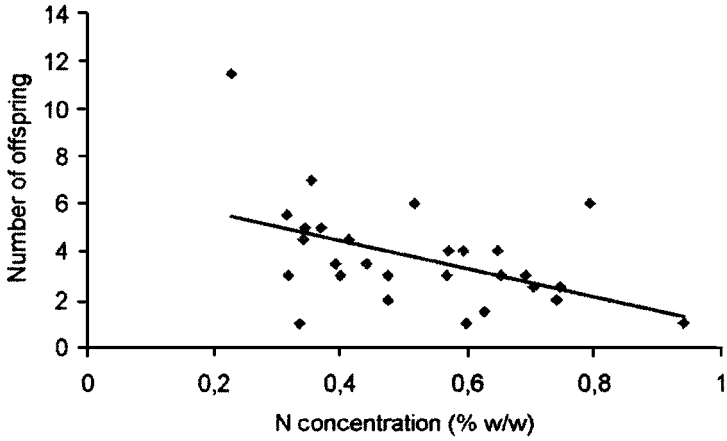

FIG. 5. Regression between N concentration (% w/w) in shoots of *Plantago lanceolata* and the number of offspring produced by *Myzus persicae* during 7 days.

the phytochemical response of plants to herbivore damage by larvae of *Junonia coenia*.

Total phytosterol, sitosterol, and campesterol content in shoots increased with N concentration. Earthworm presence increased the N concentration and the phytosterol content of *P. lanceolata* shoots when litter was mixed homogeneously into soil. The increase in phytosterols with increasing N concentration in shoots suggests that the availability of N affects the biosynthesis of phytosterols. By influencing N availability to plants, earthworms may indirectly affect the content of phytosterols in shoots. Since insects rely on phytosterols to build up ecdysteroids as molting hormones (Svoboda et al., 1994), earthworms may affect herbivore development and reproduction by indirectly changing the phytosterol content of plants. In the present study, the reproduction of *M. persicae* decreased with increasing shoot N concentration, but did not correlate with the phytosterols. Since aphids feed on phloem sap, changes in other chemical compounds transported in the phloem (e.g., amino acids) might have affected their performance.

This study documents that decomposers and the spatial distribution of litter may alter the defensive chemistry of plants. Therefore, earthworms may not only increase plant nutrient contents (Wolters and Stickan, 1991; Haimi et al., 1992; Alphei et al., 1996; Wurst et al., 2003), but also alter the defense mechanisms of plants. So far the majority of studies on plant-defensive chemistry have concentrated on the aboveground world. It is time to broaden this perspective and include the effects of belowground biota that directly (e.g., root herbivores, VAM) and indirectly (decomposers) interact with the roots.

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SEMIOCHEMICALS FROM FUNGAL ASSOCIATES OF BARK BEETLES MAY MEDIATE HOST LOCATION BEHAVIOR OF PARASITOIDS

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Abstract—In laboratory olfactometer bioassays, females of two hymenopteran parasitoid species, *Roptrocerus xylophagorum* (Ratzeburg) (Hymenoptera: Pteromalidae) and *Spathius pallidus* (Ashmead) (Hymenoptera: Braconidae), were attracted to odors from bark or bolts of loblolly pine, *Pinus taeda* L., colonized by bluestain fungi (genus *Ophiostoma*) associated with the parasitoids' bark beetle hosts. Mock-inoculated bolts and bark were less attractive or unattractive in these bioassays. Bark infested with host larvae that lacked their fungal and other normal microbial associates was attractive to *R. xylophagorum* females, but was less so than bark infested with larvae possessing their normal complement of associated microbes. In contrast, in oviposition bioassays, *R. xylophagorum* females spent approximately equal time searching, made similar numbers of oviposition attempts, parasitized similar percentages of hosts, and laid similar numbers of eggs in bark fragments infested with either associate-free or associate-bearing host larvae. Furthermore, in field bioassays using bluestain-inoculated or mock-inoculated loblolly pine bolts as sources of attractants, the numbers of parasitoids attracted by the two treatments did not differ significantly and the two treatments were less attractive than bolts naturally infested with bark beetle larvae. Whereas our laboratory olfactometer data suggest that bark beetle fungal associates may enhance attraction of some parasitoids, our bioassays with associate-free hosts indicate that associate-produced are not required for short-range host location and parasitization. In addition, our field trials indicated that long-range attraction of parasitoids to the host-fungi-tree complex is not caused simply by an interaction between bluestain fungi and tree tissues.

Key Words—Scolytidae, Pteromalidae, Braconidae, *Pinus*, *Dendroctonus*, *Ips*, parasitoids, host location, fungi, attraction, tritrophic, symbiosis.

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INTRODUCTION

The mechanisms mediating host location by the guild of hymenopteran species that parasitize the larvae of bark beetles (Coleoptera: Scolytidae) are not completely understood (Bushing, 1965; Mills, 1983; Dahlsten and Berisford, 1995). Olfactory cues are clearly important (Kudon and Berisford, 1981; Payne, 1989; Sullivan et al., 1997; Pettersson et al., 2001b), but the chemical composition of the cues and their biological origins have been only partially characterized. Evidence suggests that an interaction between bark beetles and host tree phloem results in production of host location cues that are exploited by the parasitoids. Bark excised from healthy trees does not stimulate attraction or other host finding behaviors in bark beetle parasitoids, but will do so after infestation by bark beetle larvae (Samson, 1984; Mills et al., 1991; Sullivan et al., 2000). For several parasitoid species, infested bark remains attractive after removal of hosts, whereas isolated bark beetle larvae do not elicit parasitoid attraction, arrestment, or oviposition (Mills et al., 1991; Sullivan et al., 2000).

Bark beetles are closely associated with a variety of microorganisms, particularly fungi, which grow within the beetles' gallery systems and surrounding tree tissues, and are transported to new host trees during beetle dispersal (reviewed in Paine et al., 1997). Anecdotal and circumstantial evidence suggests that bark beetle-associated fungi might be sources of parasitoid host location cues (Dahlsten and Berisford, 1995; Six and Dahlsten, 1999). Fungal symbionts of bark beetles possess qualities that make them potentially ideal indicators of host presence to parasitoids. Symbiotic fungi are closely associated with bark beetles in both time and space (Rumbold, 1931; Whitney, 1971; Bridges et al., 1984; Solheim, 1992), and the presence of preferred, late-instar beetle larvae within the bark coincides predictably with the abundance of certain fungi (Howe et al., 1971; Bridges et al., 1984; Sullivan, 1997). In addition, fungi, including those associated with bark beetles, produce a range of volatile compounds either *de novo* or from precursors present in their environment, and these compounds or compound blends can be highly specific in their chemistry (Brand et al., 1977; Brand and Barras, 1977; Leufven et al., 1988; Hanssen, 1993). However, direct experimental evidence demonstrating a role for bark beetle-associated fungi in parasitoid host location is limited (Dahlsten and Berisford, 1995; Six and Dahlsten, 1999).

The purpose of this study was to investigate the possibility that fungal associates of bark beetles mediate host location by the beetles' parasitoids. We hypothesized that if fungal associates play this role, then (1) fungal associate-colonized tree tissue should stimulate parasitoid host-seeking in the absence of bark beetles, and, conversely, (2) elimination of microbial associates should inhibit parasitoid host location behaviors directed toward bark beetle hosts. Our laboratory tests of these hypotheses were performed largely with the parasitoid *Roptrocerus xylophagorum* (Ratzeburg) (Pteromalidae) because this species was

available in large numbers and readily seeks and parasitises hosts in the laboratory. More than a dozen economically important species of bark beetle in North America and Europe are hosts for this wide ranging and abundant parasitoid (Sullivan et al., 2000). *R. xylophagorum* prefers late-instar larval hosts but also parasitises pupae (Samson, 1984). Tests of parasitoid attraction to fungi-colonized tree tissue were performed exclusively with the beetles' bluestain associates (order Ophiostomatales) because of their reliable association with bark beetles (Upadhyay, 1981) and their ability to grow relatively rapidly into tree tissues in the absence of insects (Bramble and Holst, 1940; Paine and Stephen, 1987).

METHODS AND MATERIALS

Insects for Laboratory Assays. *R. xylophagorum* were obtained from a laboratory colony maintained for several generations on bolts of loblolly pine, *Pinus taeda* L., artificially infested with the southern pine engraver, *Ips grandicollis* (Eichhoff) (Sullivan et al., 1999). *Spathius pallidus* (Ashmead) (Braconidae) females were collected as they emerged from loblolly pine bolts infested by the southern pine beetle, *Dendroctonus frontalis* Zimmermann, and/or *Ips* spp. bark beetles. Parasitoids and beetles were originally obtained in Louisiana, Georgia, Alabama, and Mississippi. After emergence, parasitoids were maintained in mixed-sex groups within 250 ml Erlenmeyer flasks held at $8 \pm 1^{\circ}$C with a 14:10 (L:D) hr light regime and were provided honey and water. Assayed *R. xylophagorum* were 5–7 day old; *S. pallidus* were 2–24 day old.

Fungi. Pure cultures of the bluestain fungi *Ophiostoma minus* (Hedgcock) H. & P. Sydow and *Ophiostoma ips* (Rumbold) Nannfeldt were obtained from perithecia within pine bark infested with either *D. frontalis* or *I. grandicollis*, respectively, and were maintained on phloem-strip agar (2% agar and loblolly pine phloem, autoclaved) slants in 50 ml screw-top culture tubes. Slants were incubated at 23–27°C until sporulating perithecia were observed. Inoculum consisted of sterile, distilled water (7–8 ml) swirled gently inside these slant tubes for several minutes.

Bolt Inoculations. Bolts (30–50 cm long, 13–25 cm diam) were cut with an ethanol-sterilized bow saw from a live loblolly pine, and the ends were coated with a 2% solution of sorbic acid and methyl paraben to retard mold growth. The outer, corky bark was shaved to remove loose bark flakes, sprayed to saturation with 70% ethanol, and allowed to dry. Bolts were inoculated by removing disks of bark (14 mm diam), placing a drop of inoculum on the exposed sapwood, replacing the disks, and covering them with masking tape. Inoculation points were spaced 5 cm apart circumferentially and 15 cm apart lengthwise. Bolts were incubated at 21–29°C and 50–70% RH.

For both the field trapping experiment and flight tunnel bioassay, the above procedure was modified slightly. Longer bolts (1–2 m) were used, and inoculum (~1 ml) was applied evenly to saw-cuts (2 mm wide) that encircled the bolts and lightly scored the sapwood. The saw-cuts were made with an ethanol-sterilized pruning saw every 35 cm along the bolts and, following inoculation, were covered by masking tape. This inoculation technique resulted in a more complete fungal colonization of the bolts than the bark disk method.

Associate-Free Hosts. Studies on the effects of elimination of bark beetle associates on parasitoid host location were conducted with *I. grandicollis* because this species can develop normally without its usual microbial associates (Yearian et al., 1972). Eggs of *I. grandicollis* were surface-sterilized in improved White's solution (Barras, 1972), rinsed repeatedly in sterile-distilled water, and allowed to hatch on moistened, autoclaved filter paper in Petri dishes. Individual, newly-hatched larvae ($\leq$1 day old) were transferred to an artificial "niche" (1 mm^3) cut into the phloem of a bark "sandwich." Bark sandwiches consisted of freshly excised pieces of loblolly pine bark (3 $\times$ 7 cm) with the exposed phloem surface pressed to a clean, sterile microscope slide (5 $\times$ 7.5 cm) (Sullivan et al., 2000). Bark for sandwiches are excised with sterile implements from bolts that had been shaved and sprayed with ethanol as described above. Sandwiches were infested with up to 10 larvae and then incubated at 29°C and ~80% RH until larvae reached the third instar. Aseptic technique was used for all steps in the preparation and handling of eggs, larvae, and bark sandwiches.

Y-Tube Bioassays with R. xylophagorum. We assayed the anemotaxis of walking female *R. xylophagorum* in a Y-tube olfactometer described in detail elsewhere (Sullivan et al., 2000). Individual females were released into the stem of a glass Y (4 mm i.d., stem 4 cm, branches 5 cm and 135° to the stem) with each branch receiving air (30 ml/min, 25–27°C and 50–70% RH) from a different sample holding tube. A parasitoid was recorded as choosing an odor source when it walked 3 cm into the associated olfactometer branch. Parasitoids that failed to make a choice during 5 min were recorded as nonresponders. The assignment of odor sources to each branch was reversed and Y-tubes were replaced with clean ones after each trial. Individual parasitoids were tested only once.

The first experiment tested female attraction to bark rectangles (3 $\times$ 7 cm, both phloem and corky bark) excised from bolts inoculated 2–3 wk earlier with *O. ips*. Bark rectangles were cut lengthwise with the grain and centered over the inoculation sites, but did not include the replaced bark disks. *O. ips* was chosen as the inoculum for this test because it is the bluestain fungus associated with *I. grandicollis*, the host on which the bioassayed *R. xylophagorum* were reared. A sterile, "mock" inoculum was produced by passing the culture rinsate inoculum through a 0.2 μm-mesh syringe filter to remove microbial propagules. Parasitoids were given three binary choices (replicated $\times$36): (A) *O. ips*-inoculated bark *versus*

clean air, (B) mock-inoculated bark *versus* clean air, and (C) *O. ips*-inoculated bark *versus* mock-inoculated bark.

The second experiment tested odors from bark sandwiches infested with 10 third instar *I. grandicollis* that were either associate-free or obtained from naturally infested bolts and, thus, presumably had their normal compliment of microbial associates ("unaltered" larvae). Infested bark sandwiches were incubated 3 day at 29°C and 70–80% RH before assays. Parasitoids were given three binary choices (replicated $\times 44$): (A) bark sandwiches with unaltered larvae *versus* clean air, (B) bark sandwiches with associate-free larvae *versus* clean air, and (C) bark sandwiches with unaltered larvae *versus* bark sandwiches with associate-free larvae. In both Y-tube experiments, component tests were balanced for day, time of day, and tree (bolts/bark were derived from up to three different trees).

A significant preference for one olfactometer branch was identified with the G-test for goodness-of-fit or, when fewer than 25 insects responded, by determining the probability of the observed outcome from a table of cumulative binomial probabilities assuming a null hypothesis of no preference (Sokal and Rohlf, 1995).

Close-Range Host Search and Oviposition Assay with R. xylophagorum. Pairs of bark sandwiches were infested with an identical number (5–10/sandwich) of either associate-free or unaltered third instar *I. grandicollis* larvae, incubated for 3 days at 29°C, and tested in random order in a double-blind bioassay. The microscope slide was removed from each sandwich, and the bark was placed phloem side-up in a 10 cm diam glass Petri dish with a single female *R. xylophagorum*. A piece of filter paper on the exposed phloem surface concealed any visual host cues. During 15 min, we recorded the number of times the female inserted her ovipositor into the filter paper (considered a single oviposition attempt) and the total time she spent searching on the bark. Observations were performed consecutively with three different females and the same sandwich/dish, and then all three females were placed into the dish at once and allowed to host-search and oviposit undisturbed for ~90 min. Afterward, the bark pieces were dissected and examined for percent parasitization of the host larvae, and the number of eggs laid per host. The experiment was replicated $\times 5$ and analyzed with a Wilcoxon signed-rank test. Samples of phloem and frass from all infested sandwiches were plated on 2% malt agar and incubated at room temperature for 4–12 days.

Field Trapping. Parasitoid attraction to bolts inoculated with *O. minus*, the bluestain associate of *D. frontalis*, was tested within active *D. frontalis* infestations. Bolts were divided into 30–35 cm long sections, and four evenly-spaced vertical strips (3 cm wide) of bark were removed from each to approximate the phloem damage and air-exposure produced by bark beetle mines. Five bait treatments were compared: a bolt section inoculated with *O. minus* and incubated either 3–4 wk (1) or 5–6 wk (2), a bolt section inoculated with sterile, distilled water ("mock"-inoculated) and incubated either 3–4 wk (3) or 5–6 wk (4), and a bolt (30–35 cm long) from a loblolly pine infested with late larval instars of *D. frontalis* (5).

An unbaited stovepipe (30 cm long, 20 cm diam) served as a "semiochemical-free" control. Bolts and stovepipes were erected on 2 m-high metal posts, covered by a bag of fine-mesh nylon screen (to prevent beetle attacks and provide a uniform visual appearance among treatments), and then surrounded by a cylinder (30 cm high, 25 cm diam) of 1.3 cm-mesh plastic hardware cloth coated with Stikem Special® (Seabright Laboratories, Emeryville, CA). Traps were arranged in randomized complete blocks, with traps spaced >3 m apart within blocks, >5 m apart between blocks, and >1.5 m from any tree. Blocks of traps were erected in the early afternoon (2–3 at one time), and screens were collected and bolts discarded after 24 hr. Eleven replicates were completed in the Homochitto National Forest, Mississippi, between 25 June and 25 July 2002. Raw catch of parasitoid females was transformed with $\log_{10}(X + 1)$ to remove heteroscedasticity and analyzed with a two-way analysis of variance (with block and treatment as factors) and the Student–Newman–Keuls (SNK) test for all-pairwise comparisons.

Flight Tunnel Bioassay with S. pallidus. We compared the relative attractiveness of *O. minus*- and mock-inoculated pine bolts to the parasitoid *S. pallidus* in a plate glass flight tunnel (40 × 40 × 100 cm long). The tunnel was operated in a greenhouse (29–35°C and 50–75% RH) with natural light filtered through a white cloth canopy (2,500–10,000 lx) and an air speed of 1 m/sec. Fifteen female *S. pallidus* were released from a screen box placed 75 cm downwind from a vertical test bolt (30 cm long), and the number of individuals exhibiting oriented flight and landing on the bolt during 20 min was recorded. Bolts inoculated 5–6 wk previously with either *O. minus* or sterile distilled water were alternated between trials. Each treatment was replicated 6–7 times, and results were analyzed with a Mann–Whitney rank sum test. All statistical analyses were performed using SigmaStat™ 2.03 software (SPSS, 1997), and an alpha level of 0.05 was used in all tests.

RESULTS

Response of R. xylophagorum to Bark Colonized by O. ips. Fifty to eighty percent of the phloem in bark rectangles excised from *O. ips*-inoculated bolts was stained black or appeared necrotic, whereas phloem from the mock-inoculated bolts was white and apparently healthy except within 1–2 mm from inoculation points. In Y-olfactometer assays, bark rectangles from *O. ips*-inoculated bolts were attractive to female *R. xylophagorum*, whereas those from the mock-inoculated bolts were not (Figure 1). When exposed to both simultaneously, female *R. xylophagorum* showed a strong preference for bark from *O. ips*-inoculated bolts over that from mock-inoculated bolts.

Response of R. xylophagorum to Associate-Free Hosts. No bacteria, yeasts, or bluestain fungi were cultured from 88 samples of frass and phloem from 16 bark sandwiches infested with associate-free *I. grandicollis* larvae. Seven of the

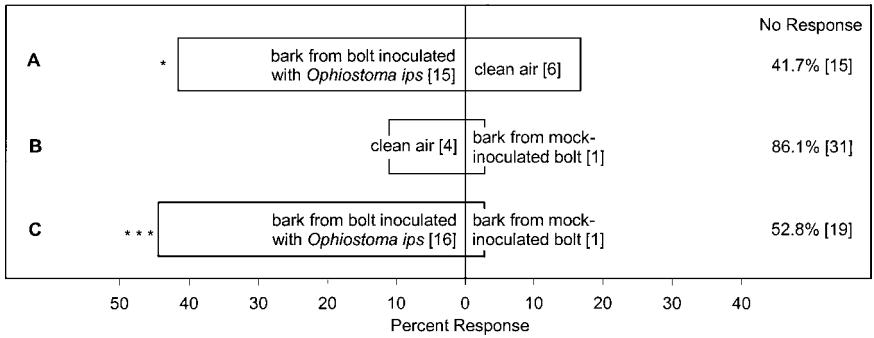

FIG. 1. Responses by individual *Roptrocerus xylophagorum* females in a Y-olfactometer to bark excised from loblolly pine bolts inoculated 2–3 wk previously either with the bluestain fungus, *Ophiostoma ips*, or filter-sterilized *O. ips* inoculum. Raw numbers of insects responding/not responding are shown in brackets. Asterisks indicate a significant preference for that treatment ($^{*}P < 0.05$; $^{***}P < 0.001$).

samples (six sandwiches) contained mycelial fungi that were either saprophytes of the corky bark of pines (DeHoog and Morgan-Jones, 1978), common molds (e.g., *Penicillium* spp.), or fungi otherwise not typically recognized as beetle associates. In contrast, all 15 sandwiches infested with unaltered beetle larvae were colonized by yeasts and/or bacteria (all 83 samples cultured) and *O. ips* (75 of 83 samples). Six of fifteen sandwiches infested with unaltered beetle larvae also had *Trichoderma* spp. present. Survival of *I. grandicollis* in bark sandwiches during the 3 day incubations was similar for both treatments ($87.5 \pm 4.1\%$ SE for associate-free *versus* $80.6 \pm 4.8\%$ for unaltered larvae; $t_{(12,13)} = 138.0$, $P = 0.34$, Mann–Whitney test), as was the apparent rate of development evidenced by the percentage that molted to pupae ($30.9 \pm 5.4\%$ for associate-free *versus* $24.3 \pm 5.4\%$ for unaltered larvae; $t_{(12,13)} = 141.5$, $P = 0.45$, Mann–Whitney test).

Bark infested with either associate-free or unaltered larvae of *I. grandicollis* was attractive to female *R. xylophagorum* in the Y-olfactometer (Figure 2). However, *R. xylophagorum* showed a strong preference for bark infested with unaltered larvae over bark infested with associate-free larvae. When allowed contact with the bark, *R. xylophagorum* females spent roughly equal time searching and made similar numbers of oviposition attempts on bark infested either with associate-free or unaltered hosts (Table 1). There were also no significant differences between the treatments in either percentages of parasitized hosts or the number of eggs laid per host.

Field Response of Parasitoids to Bolts Inoculated with O. minus. Approximately 80–100% of the phloem/sapwood interface on bolts inoculated with *O. minus* either 3–4 or 5–6 wk previously was covered by dark stain and perithecia of this fungus. Cross sections of these bolts revealed extensive penetration of stain

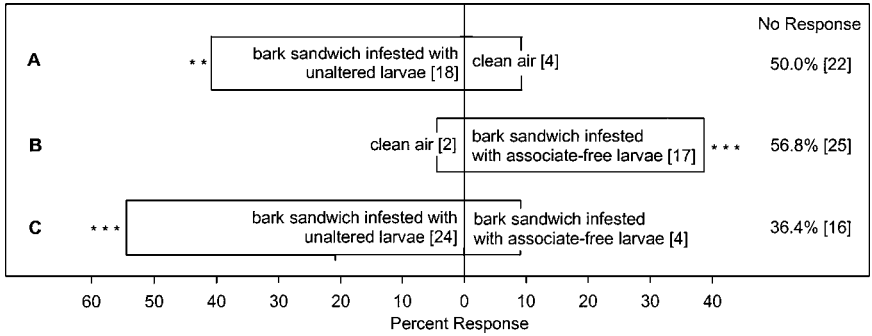

FIG. 2. Responses by individual *Roptrocerus xylophagorum* females in a Y-olfactometer to bark sandwiches infested artificially with third instar *Ips grandicollis* larvae that were either obtained from a naturally infested pine bolt (and ostensibly possessed their normal microbial associates) or reared from surface-sterilized eggs (and thus lacked associates). Raw numbers of insects responding/not responding are shown in brackets. Asterisks indicate a significant preference for that treatment ($^{**}P < 0.01$; $^{***}P < 0.001$).

into the sapwood. In contrast, the phloem and sapwood of mock-inoculated bolts generally appeared healthy, and discoloration and other evidence of necrosis were minimal or absent. Total catch of *D. frontalis* parasitoids was significantly greater at traps baited with either 5–6-wk-old *O. minus*-inoculated bolts or host-infested bolts, than unbaited stovepipe traps ($P = 0.004$ and $P < 0.001$, respectively, SNK test) (Figure 3). However, *O. minus*-inoculated bolts did not catch significantly more parasitoids than mock-inoculated bolts incubated the same length of time. When parasitoid species were analyzed individually, *S. pallidus* was the only species caught in greater numbers on an inoculated bolt treatment (i.e., 5–6-wk-old *O. minus*-inoculated) than on the unbaited controls ($P = 0.033$, SNK test),

TABLE 1. HOST SEARCHING AND OVIPOSITION RESPONSES OF FEMALE *Roptrocerus xylophagorum* TO BARK SANDWICHES INFESTED 3 DAYS PREVIOUSLY WITH THIRD INSTAR LARVAE OF ITS HOST, *Ips grandicollis*

	Axenic host larvae	Untreated host larvae
Live hosts per sandwich	8.0 ± 0.9	7.2 ± 0.9
Ratio pupal/larval hosts	0.27 ± 0.05	0.45 ± 0.22
Search time (min)[a]	7.7 ± 1.9	7.0 ± 1.7
Oviposition attempts[a]	2.9 ± 0.7	2.5 ± 0.8
Percent parasitization (all hosts)	54.7 ± 9.9	50.0 ± 6.6
Percent parasitization (larvae)	66.6 ± 14.0	71.9 ± 12.1
Eggs per host	1.30 ± 0.11	1.60 ± 0.04

[a] Mean female response per bark sandwich.

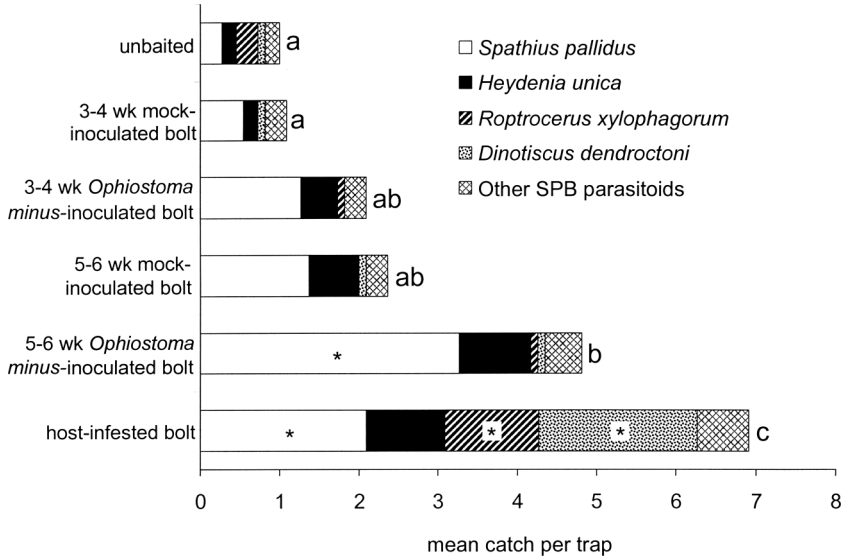

FIG. 3. Responses of female parasitoids of *Dendroctonus frontalis* to sticky traps placed within an active *D. frontalis* infestation. Traps were baited with loblolly pine bolts that had been either inoculated with *O. minus* (the bluestain fungus associated with *D. frontalis*), inoculated with sterile distilled water ("mock-inoculated"), or infested naturally with late-instar *D. frontalis* larvae. Fungus- and mock-inoculated bolts had been incubated at 21–29°C for either 3–4 or 5–6 wk. "Other SPB parasitoids" included *Coeloides pissodis*, *Dendrosoter sulcatus*, *Eurytoma tomici*, and *Eupelmus* sp. Treatments associated with the same letter were not significantly different in catch of all *D. frontalis* parasitoids ($\alpha = 0.05$). Asterisks indicate when an individual parasitoid species was attracted to a particular bait treatment in significantly greater numbers than to the unbaited control trap.

and no species was trapped in higher numbers on fungus-inoculated bolts than mock-inoculated bolts. Three species, *S. pallidus*, *R. xylophagorum*, and *Dinotiscus dendroctoni* (Ashmead) (Pteromalidae), were attracted to *D. frontalis*-infested bolts in greater numbers than the unbaited controls. Other hymenopteran parasitoids of *D. frontalis* trapped in small numbers included *Heydenia unica* Cook and Davis (Pteromalidae), *Coeloides pissodis* (Ashmead) (Braconidae), *Dendrosoter sulcatus* Muesbeck (Braconidae), *Eurytoma tomici* Ashmead (Eurytomidae), and *Eupelmus* sp. (Eupelmidae).

Flight Tunnel Response by S. pallidus to O. minus-Inoculated Bolts. In the flight tunnel, a significantly higher percentage of *S. pallidus* females were stimulated to fly upwind and alight on 5–6-wk-old bolts inoculated with *O. minus* than mock-inoculated bolts of the same age ($t_{(6,7)} = 59.5$, $P = 0.008$) (Figure 4).

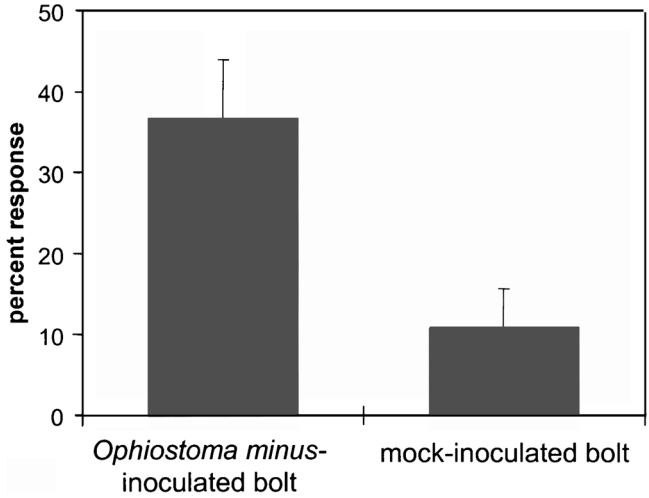

FIG. 4. Percentage of *Spathius pallidus* females responding in single-choice flight tunnel tests to loblolly pine bolts inoculated 5–6 wk previously with either *O. minus* or sterile distilled water. Responding females flew toward and landed on vertically oriented bolts positioned 75 cm upwind from the parasitoids' release point. Responses to the two treatments were significantly different ($P = 0.008$, Mann–Whitney rank sum test).

DISCUSSION

Results from the laboratory bioassays suggested that bole tissue of loblolly pines produced semiochemical attractants for two bark beetle parasitoids, *R. xylophagorum* and *S. pallidus*, after inoculation with the bluestain fungi *O. ips* and *O. minus*, respectively. In light of the close association between these fungi and hosts of the parasitoids, the results suggest that olfactory cues produced by bluestain fungi may provide at least some of the cues that *R. xylophagorum* and *S. pallidus* use to locate host habitats. Parasitoids of other insects, notably siricid woodwasps and drosophilid and tephritid fruit flies, also have been shown to respond to semiochemical cues produced by microbial associates of their hosts (Madden, 1968; Spradberry, 1970; Dicke, 1988; Thibout et al., 1993). Natural selection tends to favor adaptations in host insects that reduce apparency to parasitoids (Vet et al., 1991), and such selective pressures may explain the apparent lack of host location cues produced by bark beetle larvae isolated from tree tissue (Mills et al., 1991; Sullivan et al., 2000). In contrast, microbial symbionts of bark beetles are under no direct selective pressure to limit their apparency to the beetles' natural enemies, hence cues arising from the host's symbionts may serve as surrogates for host-produced cues.

Some symbionts of bark beetles, particularly the bluestain fungi, reduce the fitness and survival of bark beetle broods by competing directly with the beetles for nutrients or otherwise rendering bark unsuitable for brood development (Barras, 1970; Yearian et al., 1972; Fox et al., 1993; Six and Paine, 1998). Our data suggest the possibility that these fungi may reduce brood survival further by attracting natural enemies of beetle larvae.

However, cues from microbial associates of *I. grandicollis* were not necessary for short-range host location and parasitization by *R. xylophagorum*, suggesting that associate-generated attractants might play a restricted or unessential role in mediating these behaviors. In laboratory bioassays, bark infested with *I. grandicollis* larvae that lacked the normal complement of microbial associates still stimulated attraction, arrestment, ovipositor drilling, and oviposition by *R. xylophagorum*. Nonetheless, in the Y-olfactometer, *R. xylophagorum* showed a strong preference for bark infested by associate-bearing larvae over bark infested with associate-free larvae. In combination, these results suggest that cues from host-associated microbes are not essential for successful host finding, but nevertheless may enhance host detectability or otherwise influence host location by *R. xylophagorum*.

In the field experiment, parasitoids failed to discriminate between bluestain- and mock-inoculated bolts, and bluestain-colonized bolts were less attractive than host-colonized bolts. This suggests that long-range host location cues arising from the host-fungi-tree complex are not produced simply by an interaction between bluestain fungi and tree tissues. *R. xylophagorum's* apparent lack of attraction in field tests to pine bole tissue colonized by the bluestain fungus *O. minus* contrasted with their attraction in Y-olfactometer tests to tissue colonized by another bluestain fungus, *O. ips*. This was unexpected given that these fungi are similar in biology and both are associated with hosts for *R. xylophagorum*. Flying *R. xylophagorum* may not respond to bluestain-associated odors the same as walking individuals, or odors from excised, bluestain-colonized bark (used in the *O. ips* tests) may produce a different response than odors from whole, bluestain-colonized bolts (used in the *O. minus* tests). Alternatively, in the Y-tube bioassays, the wasps were presented with a limited number of stimuli to which they could respond, whereas in bioassays run under field conditions, wasps were presented with a plethora of olfactory, visual, and tactile sensory inputs.

In the present study, parasitoid-attracting semiochemicals may have been produced by tissue of the fungus itself or, alternatively, by tree tissue in response to fungal infection. Bark beetle-associated fungi produce a variety of distinctive compounds when grown in pure culture (Brand et al., 1977; Brand and Barras, 1977; Leufven et al., 1988; Hanssen, 1993). In addition, infection of conifers by a variety of bark beetle-associated fungi (particularly *Ophiostoma* spp.) generates blends of volatiles in the surrounding tissue that differ quantitatively and qualitatively from those associated with healthy tissue (Russell and Berryman, 1976; Raffa and Berryman, 1982; Cook and Hain, 1985; Paine et al., 1987; Raffa and

Smalley, 1995). Studies on the chemistry of the host location cues of bark beetle parasitoids have demonstrated the importance of a group of approximately 7–15 cyclic monoterpene alcohols, ketones, and aldehydes. These oxygenated monoterpenes are consistently associated with conifer tissues infested with bark beetle larvae (Birgersson et al., 1992; Sullivan et al., 2000; Pettersson, 2001b), elicit strong antennal responses from several parasitoids of conifer-infesting bark beetles (Salom et al., 1991, 1992; Pettersson et al., 2000, 2001a,b; Pettersson, 2001a), and, in various combinations, are attractive to female parasitoids (Pettersson, 2001a; Pettersson et al., 2000, 2001a). Necrotic phloem surrounding sites on pine bolts inoculated with *O. minus* or *O. ips* contain elevated levels of 6–8 of these oxygenated monoterpenes (Sullivan, 1997), and bark beetle-associated yeasts and mycelial fungi grown in pure culture with conifer-derived substances also produce some of these compounds (Leufven et al., 1988; Sullivan, 1997). Hence, bark beetle-associated fungi may elicit parasitoid attraction by increasing the concentrations of parasitoid-attracting oxygenated monoterpenes within tree tissues colonized by bark beetles.

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DIFFERENCES AMONG ANTIMICROBIAL PROPERTIES OF CARRION BEETLE SECRETIONS REFLECT PHYLOGENY AND ECOLOGY

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Abstract—Carrion beetles (Coleoptera: Silphidae) consist of two subfamilies in North America. Members of the Silphinae arrive at carcasses during the mid-stage of decay and their larvae feed on developing maggots, while members of the Nicrophorinae bury and tend carcasses upon which their developing larvae feed. The Nicrophorinae maintain the condition of the carcass by applying oral and anal secretions that reduce carcass decay apparently through bacterial inhibition, although quantification has not been made. We hypothesized that enzymes in the oral and anal secretions of the subfamily Nicrophorinae would inhibit bacterial growth, while secretions from the subfamily Silphinae would not. The secretions were assayed for inhibitory effects with a Microtox Analyzer that monitors the decrease in bioluminescence from the bacterium *Vibrio fischerii*. We found a significant difference of bioluminescence in the control compared to secretions of 8 out of 10 tested Nicrophorinae (with oral secretions being most active), while only anal secretions from *Necrodes surinimensis* of the Siphinae significantly reduced bacterial survival. These data follow the known phylogenic relationship in which *Necrodes* is the closest genus to the Nicrophorinae. The two species of Nicrophorinae, which did not show significant reductions in bacterial growth, differ ecologically from the others. Thus, the presence of antimicrobial compounds in most Nicrophorinae secretions, but not in most other Silphinae, represents an adaptation to preserve the buried carcass.

Key Words—*Nicrophorus*, burying beetle, antimicrobial, carrion beetle, Silphidae.

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INTRODUCTION

When an organism dies, an intense competition begins among vertebrate scavengers, invertebrate necrophores including fly larvae and beetles, and bacterial and fungal decomposers for the resources contained in its body tissue. Among the attracted species are the carrion beetles (Coleoptera: Silphidae) consisting of six genera. Members of five genera, belonging to the subfamily Silphinae, tend to arrive at a carcass during the midstages of decay and feed upon both the carcass and developing fly larvae (Ratcliffe, 1972, 1996). In contrast, burying beetles consisting of beetles in the genus *Nicrophorus* have a unique reproductive strategy.

Nicrophorus beetles reproduce by burying a small vertebrate carcass and molding it into a brood ball upon which they lay eggs and rear their young. These beetles are unique among insects in conducting bi-parental care, and many experiments have shown this care is necessary for successful rearing of larvae (e.g., Scott, 1998). Numerous studies have also reported that brood balls show little evidence of microbial decay and associated odor (Trumbo, 1994; Ratcliffe, 1996; Suzuki, 1999). There are a high number of opportunistic soil-dwelling microorganisms that come in contact with the carcass after it has been buried, and carrion beetles transport a high number of bacterial and fungal species between carrion sources (Solter et al., 1989; Berdela et al., 1994). Although the mechanism by which burying beetles achieve the observed reduction in carcass decomposition is unknown, parental beetles maintain the brood ball by continually covering it with anal and oral secretions (Milne and Milne, 1976; Ohkawara et al., 1998; Scott 1998), leading several authors to suggest that these secretions are antimicrobial (Trumbo, 1994; Ratcliffe, 1996; Scott, 1998).

The burial behavior, bi-parental care, and use of secretions to potentially reduce bacterial growth among the Nicrophorinae are unique among the insects and reflect a highly evolved reproductive strategy (Scott, 1998). Differences exist among the Nicrophorinae in ecology, brooding behavior, and carcass size preference (reviewed by Scott, 1998) that may result in differences in antimicrobial properties, as observed in other closely related vertebrate and invertebrate species (Zasloff, 2002).

Compared to the Nicrophorinae, relatively little is known about the biology and ecology of the Silphinae (Anderson and Peck, 1985; Ratcliffe, 1996). These beetles tend to use larger carcasses, although they also compete with *Nicrophorus* species for small carcasses that they use for food but not larval development (Bishop, 2001). Physiological characterization of defensive secretions from *Necrodes surinimensis* (Eisner and Meinwald, 1982) and oral secretions from *Nicrophorus marginatus* (Rana et al., 1997) show enzymes with the potential to reduce bacterial growth in both groups of beetles. We hypothesized that the Nicrophorinae would possess antimicrobial compounds in their secretions, while we expected the Silphinae to have few antimicrobial compounds.

METHODS AND MATERIALS

Insect Collection. Adult carrion beetles were collected as needed in south-central Nebraska, USA, by using pitfall traps baited with carrion (decaying rat carcasses), following the trapping protocol of Bedick et al. (1999). Beetles were maintained in the laboratory in aquaria with moist sand at room temperature and were fed a mixed diet of fish, mammal, and bird carrion. Seven *Nicrophorus* species and one species from each of three other genera: *Necrophila*, *Necrodes*, and *Thanatopholes* were used. Voucher specimens, other than the federally endangered *Nicrophorus americanus*, were collected and deposited in the University of Nebraska at Kearney insect collection. All *N. americanus* were photographed.

Secretion Collection. When disturbed, most species of carrion beetles regurgitate, defecate, or produce anal defensive sprays (Eisner and Meinwald, 1982; Scott, 1998). We collected oral and anal secretions approximately every other day from individuals of the test species. A sterile cotton swab was placed between the mandibles or against the anus of individual beetles while gentle pressure was applied to the beetle's thorax and abdomen. Secretions were separately collected from 30 individuals into 3 ml of Tris buffer. For two rare species, *Nicrophorus pustulatus* and *N. americanus*, secretions were collected from 10 beetles into 1 ml of Tris buffer. For the protected *N. americanus*, beetles were collected from pitfall traps and secretions were collected in the field after which the beetles were released. Secretions were kept on ice for transport to the laboratory. Oral secretions are pH 5 and anal secretions are pH 9, however, preliminary assays revealed similar results at pH 7 for both secretions (Bishop, 2001). Because of Microtox protocols, secretions were collected and stored in 0.1 M Tris buffer at pH 7. Among the Silphinae, *N. surinimensis* produced only anal secretions, thus, oral secretions could not be tested. Secretions were filter sterilized with a 0.22 μm syringe filter and either used fresh or stored at –20°C for later use. Freezing resulted in no detectable decline in secretion activity (Bishop, 2001).

Toxicity Analysis. Samples of anal and oral secretions from all species were assayed by using a Microtox Model 500 Analyzer (SDI, Newark, DE). This device measures the inhibitory effects of insect secretion by monitoring the decrease in bioluminescence that is a measure of reduction in *Vibrio fischerii* numbers. Collected secretions were assayed by using the 2% screen test to measure the acute activity of samples at 5 and 15 min, following the procedure in the MicrotoxOmni™ Software from the manufacturers (AZUR Environmental, Newark, DE). As a positive control, zinc sulfate (0.01%) was used in all experiments as recommended by the manufacturer. All tests were performed in triplicate and repeated on three separate occasions. Differences among mean values were tested with a one-way analysis of variance followed by a Tukey test ($P < 0.05$).

Tritiated Thymidine Uptake Assay. As a further test of antimicrobial activity, oral and anal secretions of *N. marginatus*, the most common burying beetle

collected in Nebraska were incubated with *Bacillus cereus*, a common soil bacterium, following a modified procedure of Freshney (1994). *Bacillus cereus* was grown at 30°C overnight in Luria broth (LB) with agitation. After 16 hr, 10 μl of these cells and 100 μl of either anal secretion at pH 9 or oral secretion at pH 5 were added to 1 ml of LB with tritiated thymidine. Cells were incubated for an additional 3 hr at 30°C with agitation, after which the cells were pelleted and washed with cold 0.85% saline followed by an ice-cold 10% trichloracetic acid wash. One-half milliliter of 1% SDS and 0.3 N NaOH solution were added for 15 min at room temperature to lyse the cells. Samples were centrifuged and added to 3.5 ml of scintillation fluid and counted by using a Model LS6500 scintillation counter (Beckman Coulter Inc., Fullerton, CA). Samples were measured in triplicate, and the assay was repeated three times. Results were analyzed with a one-way analysis of variance followed by a Tukey test ($P < 0.05$) where differences were detected.

Denaturing PAGE Analysis of Anal and Oral Secretion Samples. Anal secretion of 250 μl and oral secretion of 75 μl from *N. marginatus* were precipitated in 2 ml of ice-cold acetone. The samples were stored at −20°C for 5 min and pelleted at 14,000 rpm for 2 min. The acetone was poured off, and the pellets were allowed to dry for approximately 2 min. The protein pellets were resuspended in 20 μl of SDS sample buffer and placed at 95°C for 5 min. Samples were then loaded onto 15% polyacrylamide gel, Tris-HCl, and run under denaturing conditions at 100 V. Proteins were detected with Coomassie Brilliant Blue staining.

Characterization of the Antimicrobial Agent. To investigate the nature of the active agent, the oral secretion from *N. marginatus* was subjected to either pronase treatment (10 mg/ml for 6 hr at 37°C) or heat treatment (boiling for 5 min). These treatments inactivate proteins and, thus, indicate whether or not the active agent in the secretions is proteinaceous. Ten microliters of the treated samples were then run in the Microtox assay to establish changes in antimicrobial activity.

RESULTS

Microtox Assay. Oral secretions from five of the seven tested *Nicrophorus* produced significant ($P < 0.001$) reductions in bacterial numbers after 15 min (Figure 1). Oral secretions from *Nicrophorus carolinus* and *N. pustulatus* did not reduce bacterial numbers. Among the species tested, both *Nicrophorus tomentosus* and *Nicrophorus orbicollis* possessed anal secretions that also reduced bacterial numbers (Figure 1). Only the anal secretions of one member of the Silphinae, *Necrodes surinamensis*, reduced bacterial numbers (Figure 2).

Tritiated Thymidine Uptake. The ability of oral and anal secretions from *N. marginatus* to inhibit the growth of *B. cereus* was quantified by incorporation of tritiated thymidine. This experiment served as a positive control. The anal secretion

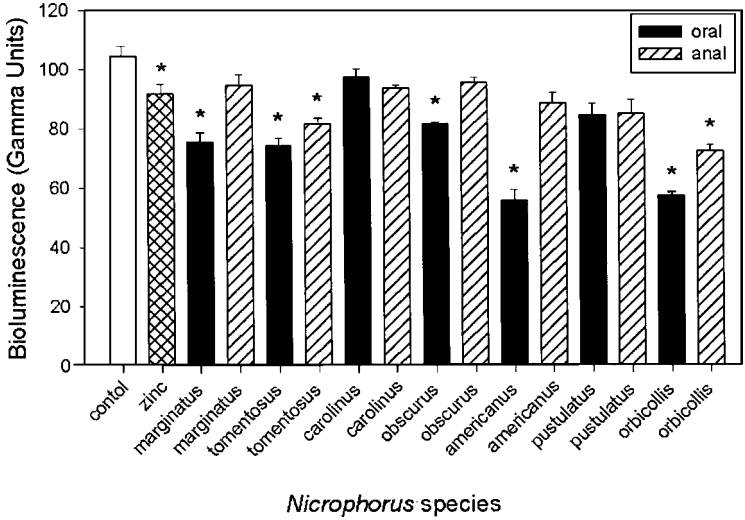

FIG. 1. Bioluminescence of the bacterium *Vibrio fischerii* when grown with anal or oral secretions from seven *Nicrophorus* species using the Microtox acute assay at 15 min. Zinc sulfate was used as a positive control. Bars indicate mean ± SE; * indicates $P < 0.05$ when compared to control ($N = 3$).

and pH 9.0 buffer were not significantly different from the control (Figure 3). Oral secretions decreased thymidine uptake by approximately 40% compared to both the control ($P = 0.039$) and the pH 5.0 buffer ($P = 0.016$).

PAGE Analysis of Secretions. Proteins from oral and anal *N. marginatus* secretions were separated by SDS PAGE. Most proteins were 35.5 kD or less (Figure 4) with oral secretions having three or potentially four bands not present in anal secretions. There also appears to be a high concentration of short peptides present in the oral secretion (Figure 4).

Characterization of the Antimicrobial Agent. The antimicrobial agent in the oral secretion was tested for sensitivity to heat and pronase with the Microtox assay to determine if it was a protein. All treatments were significantly different from control. Compared to the untreated *N. marginatus* oral secretion, treatment with pronase decreased activity by 32.5% and heat treatment decreased activity by 65% (Figure 5).

DISCUSSION

Numerous authors have described changes in the rate of carcass decomposition associated with treatment by burying beetle oral and anal secretions (see Scott,

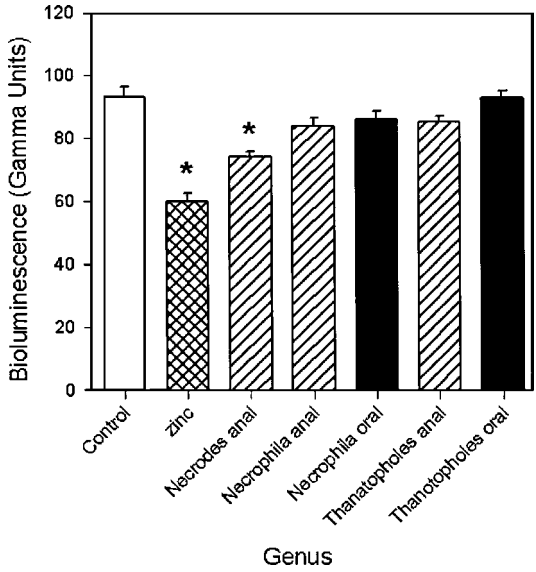

FIG. 2. Bioluminescence of the bacterium *Vibrio fischerii* when grown with anal or oral secretions from four genera of Silphinae using the Microtox acute assay at 15 min. Zinc sulfate was used as a positive control. Bars indicate mean ± SE; * indicates $P < 0.05$ when compared to control ($N = 3$).

1998). As predicted, our results demonstrate that the majority of tested *Nicrophorus* species have secretions with antimicrobial properties. The active substances primarily occur in oral secretions and are effective in reducing microbial activity by 25–40% in acute Microtox assays (Figure 1). Somewhat surprisingly, there were differences among the *Nicrophorus* species. Two of the tested species, *N. pustulatus* and *N. carolinus*, showed no antimicrobial activity, and two species, *N. tomentosus* and *N. orbicollis*, had anal secretions that reduced microbial activity (Figure 1).

The lack of antimicrobial secretions in *N. pustulatus* is likely explained by the suggested brood parasitism behavior of this species (see Ratcliffe, 1996). Field observations and laboratory data show *N. pustulatus* to be rarely collected in pitfall traps baited with carcasses despite producing substantially more offspring than other *Nicrophorus* species (Ratcliffe, 1996). Additionally, Bishop (2001) showed this species is collected more often from carcasses prepared by *Nicrophorus* beetles than from control carcasses. Thus, it appears that *N. pustulatus* utilizes carcasses that have already been treated with antimicrobial secretions from another species of burying beetles.

Nicrophorus carolinus is a relatively large carrion beetle that occurs primarily in areas with sandy soils (Bishop et al., 2002), and its lack of antimicrobial

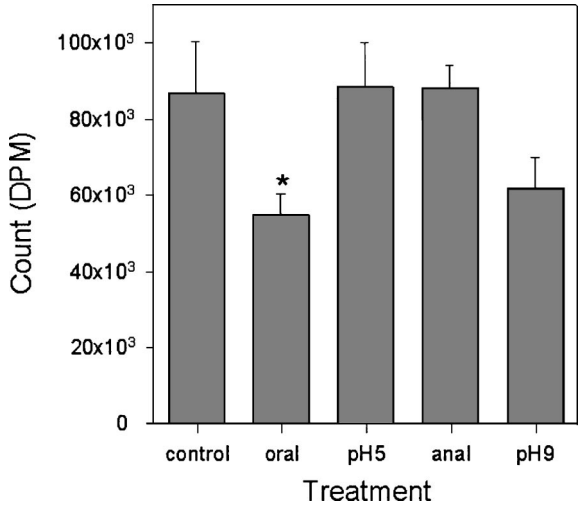

FIG. 3. Ability of *Bacillus cereus* to incorporate tritiated thymidine in buffer (control = pH 7, pH 5, pH 9) and in buffer with oral secretions (pH 5) or anal secretions (pH 9) from *Nicrophorus marginatus*. Bars indicate mean ± SE; * indicates $P < 0.05$ when compared to control ($N = 3$).

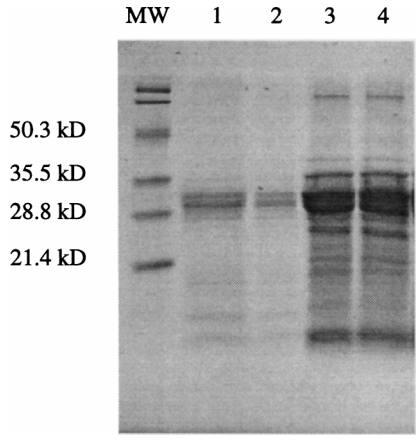

FIG. 4. A representative gel of proteins from *Nicrophorus marginatus* oral and anal secretions separated by SDS PAGE. Lane MW was low range prestained SDS PAGE standards (Bio-Rad, Hercules, CA). Lanes 1 and 2 were 250 μl anal secretions, and Lanes 3 and 4 were 75 μl oral secretions.

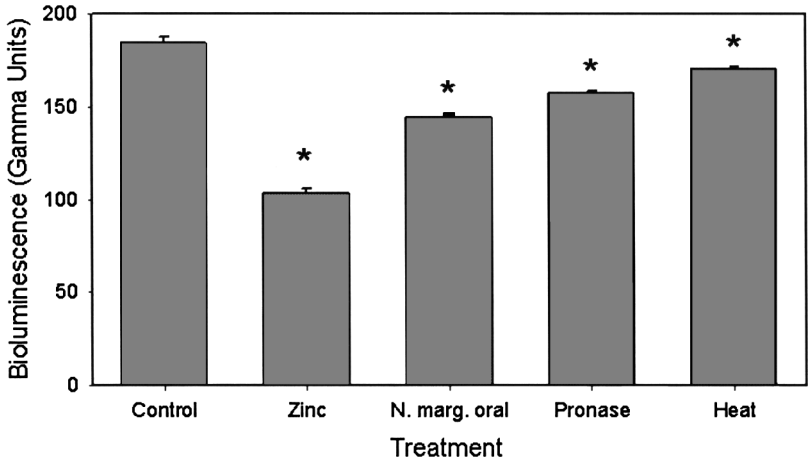

FIG. 5. Bioluminescence of the bacterium *Vibrio fischerii* when grown with oral secretion from *Nicrophorus marginatus* or oral secretion subjected to pronase treatment (10 mg/ml for 6 hr at 37°C) or heat treatment (boiling for 5 min) and measured using the Microtox assay. Bars indicate mean ± SE.; * indicates $P < 0.05$ when compared to control ($N = 3$).

secretions is more difficult to explain. Large size alone does not appear to explain the results since both *N. americanus* and *N. obscurus* are as large as or larger than *N. carolinus*, and both produce antimicrobial oral secretions. Habitat association does not appear to explain the lack of antimicrobial properties since *N. marginatus* also prefers sandy soils. Protein concentration in the oral secretions does not explain the results as we found concentrations of proteins in oral secretions to be similar between *N. carolinus* and *N. marginatus* (data not shown). Phylogenically, *N. carolinus* is most similar to *N. marginatus* and *N. obscurus* (Dobler and Muller, 2000; Szalanski et al., 2000), two species that produce antimicrobial secretions. Thus, it appears that some other undiscovered aspect of *N. carolinus* biology may explain the observed lack of antimicrobial secretions.

The presence of antimicrobial properties in the anal secretions of *N. tomentosus* and *N. orbicollis* is surprising. *N. tomentosus* is the smallest species of *Nicrophorus* tested, and its small size has ecological implications. First, it may not be able to bury the carcass as deeply as other species. Second, the production of secretions may be limited, requiring the use of both oral and anal secretions. This possibility also might explain why its secretions produced the greatest antimicrobial effects among species tested (Figure 1). In contrast, *N. orbicollis* is a medium-sized beetle associated with wet areas and loamy soils (Bishop et al., 2002). These moister soils allow greater bacterial activity and may require both oral and anal antimicrobial secretions. *N. tomentosus* and *N. orbicollis* are phylogenetically closely related and distinct from the other *Nicrophorus* species (Szalanski et al., 2000), which may explain the anal antimicrobial properties.

Among the carrion beetles in the subfamily Silphinae, only one genus produced antimicrobial secretions (Figure 2). Phylogenetically, *Necrodes* is most closely related to *Nicrophorus* (Dobler and Muller, 2000). Its defensive anal secretions have been characterized (Eisner and Meinwald, 1982) and contain a volatile oil, necrodol. Although antimicrobial, their use as a preservative seems unlikely because *N. surimensis* arrives at large (>500 g) carcasses and its larvae feed on maggots. The anal defensive secretions may represent the first step in the evolution of antimicrobial secretions for carcass preservation. The subsequent loss of effectiveness of anal secretions and evolution of effective oral secretions could have occurred because of feeding that involves preoral digestion using enzymes including PLA_2 by the Nicrophorinae (Rana et al., 1997).

Antimicrobial compounds in insect saliva have been previously reported by Lamberty et al. (2001). They reported small antimicrobial peptides that were constitutively produced in the salivary glands of the termite, *Pseudacanthotermes spiniger*. These termites are thought to cover their eggs with saliva, which protects them from bacteria and fungi. The secretions of *Nicrophorous* species may function in a similar manner. Another protein suggested to act in carcass preservation is Phospholipase A_2 (Rana et al., 1997).

Four classes of antimicrobial peptides—proline-rich, glycine-rich, cercopins, and defensins—have been identified from insects (Hetru et al., 1998). Most of these compounds are associated with insect immune response and are released internally from fat body tissues after injury. Defensins typically affect gram-positive bacteria although some activity against gram-negative species has been reported. Defensins have been recorded from seven orders of insects including Coleoptera (Hetru et al., 1998) and are the only known example of externally released antimicrobial peptides (Lamberty et al. 2001).

Because of the diversity of microorganisms found in association with carcass decay, we expect that burying beetles possess a combination of these classes of peptides or another compound that is active against gram-negative and gram-positive bacteria, and fungi. Our ability to reduce, but not entirely eliminate, antimicrobial activity through chemical and heat-treatment (Figure 5) suggests that multiple compounds are expressed as in most multicellular organisms (Zasloff, 2002). If the antimicrobial compound is a peptide, it may show resistance to heat treatment because of the ability to refold after removal from the heat. The proteins present in the secretions could also be glycosylated or lipid-associated, making them less susceptible to pronase.

Our results relate to broader issues in evolutionary biology by interlinking behavior, physiology, and ecological distribution among related species. Nicrophorinae prevent carcass decomposition by using highly evolved bi-parental care that includes carcass burial and preparation with antimicrobial compounds. By comparison, the Silphinae larvae use larger carcasses and feed on fly larvae and do not demonstrate these adaptations. Observed differences in secretion properties may be related to differences in habitat choice or behavior among closely related

species, potentially accounting for both the lack of antimicrobial properties of *N. carolinus* and the presumed brood parasitism of *N. pustulatus*. Future work characterizing the nature of the antimicrobial proteins and explaining the reasons for differences among species should reveal greater insights into the role of ecology, physiology, and behavior in speciation and ecology of this group.

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QUINONE MIXTURE AS ATTRACTANT FOR NECROPHAGOUS DUNG BEETLES SPECIALIZED ON DEAD MILLIPEDES

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Abstract—2-Methyl-1,4-benzoquinone (toluquinone) and 2-methoxy-3-methyl-1,4-benzoquinone are the most common components of defensive secretions of juliform millipedes (Diplopoda: Juliformia). A natural and a synthetic millipede-defensive secretion composed of these two substances attract dung beetles of a few *Onthophagus* species (Coleoptera: Scarabaeidae) that feed mainly on freshly dead millipedes. This olfactory mechanism and adaptation to the toxic effects of quinones enables them to be the first and exclusive users of this resource.

Key Words—Quinones, attractants, diplopod-defensive secretions, benzoquinones, dung beetles, necrophagy, Diplopoda, Scarabaeidae.

INTRODUCTION

1,4-Benzoquinones are the main components of defensive secretions of various groups of Arachnida and Insecta as well as of juliform millipedes (Diplopoda: Juliformia, orders Spirostreptida, Spirobolida, and Julida) (Blum, 1981; Huth, 2000). They are effective repellents for both invertebrate and vertebrate predators (Eisner et al., 1978; Weldon et al., 2003) and may take up more than 1% of the total body weight of a millipede (Demange, 1993; Huth, 2000). Even predators specialized on juliform millipedes may follow sophisticated strategies to avoid the quinones (Eisner et al., 1998). Because of the strong quinonoid smell of freshly

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dead Juliformia, they are unattractive for most necrophagous animals. However, in tropical regions, some dung beetles regularly feed on this resource (Cambefort, 1984; Krell et al., 1997; Krell, 1999; Brühl and Krell, 2003). They even copulate in the millipede carcasses, bring them in parts into their nest burrows, and use them as food for their offspring (personal observation).

We found that the defensive secretions of diplopods serve as an attractant for the beetles of some of these species (Krell et al., 1997, 1998; Krell, 1999; Brühl and Krell, 2003). In this study, we present further results on the attractive effect of defensive secretions of the millipede *Pelmatojulus tigrinus* (Hoffman and Mahsberg) (Spirobolida: Pachybolidae), analyze their chemical composition, and test whether benzoquinones are actually the attractive components.

METHODS AND MATERIALS

Study Area. We conducted our experiments from 1996 to 1998 in the Parc National de la Comoé in the north-eastern Côte d'Ivoire (Ivory Coast, West Africa) around the research camp of Würzburg University (Lola-Camp). This area is at the border between Guinea and Subsudan savanna attributed by Porembski (1991) to the former and by, e.g., Poilecot (1991), to the latter. Traps were set in the gallery forest of the Comoé river around the camp (3°48′58″W, 8°45′07″ − 14″N) and in the savanna between the Lola creek and the Kongo river (3°45′58″W, 8°46′20″N). Two traps were set in the wet gallery forest of the Iringou river (3°46′16″W, 8°50′21″N), but included in the data set of the Comoé gallery forest (Table 1), because the studied millipede and beetle species occur in both habitats.

Traps. We used pitfall traps made of a blue (1996) or transparent (1997, 1998) plastic funnel (about 10 cm diam.; polyethylene) placed on the top of a transparent plastic cup (polystyrene) without conservation fluid. The bait was placed beneath the funnel at the bottom of the cup. Although no rain cover was installed, no trap suffered from flooding.

Bait. (a) Diplopod-defensive secretions: We used uninjured individuals of the large millipede *Pelmatojulus tigrinus* (Hoffman and Mahsberg, 1996) (Spirobolida: Pachybolidae) as donors of the defensive secretions. Rubbing the diplopods with toilet paper induced the animals to emit their defensive secretions. Such paper soaked by the secretions was used for the baiting experiments. In the control traps, we used unsoaked toilet paper. *P. tigrinus* is the most abundant diplopod species in the gallery forest of the Iringou river near our study area and forms a small population directly in the study area in the Comoé gallery forest as well (Mahsberg, 1997).

(b) Chemicals: The following substances were tested as attractants, sometimes dissolved in ethanol p.a. (Riedel de Haën, Germany, product number 32221): 1,4-benzoquinone, >98% (Fluka, Buchs, Switzerland, 12310; lot 341571 595

TABLE 1. NUMBERS OF INDIVIDUALS OF *Onthophagus latigibber* CAPTURED BY PITFALL TRAPS BAITED WITH DIFFERENT QUINONES IN THE GALLERY FOREST OF THE COMOÉ RIVER, COMOÉ NATIONAL PARK, IVORY COAST

Baits	Number of traps	Number of specimens of *Onthophagus latigibber*
1,4-Benzoquinone	29	0
2-Methyl-1,4-benzoquinone	16	0
1,4-Benzoquinone + 2-methyl-1,4-benzoquinone	2	0
2-Methoxy-3-methyl-1,4-benzoquinone	7	2
2-Methoxy-3-methyl-1,4-benzoquinone + 2-methyl-1,4-benzoquinone	12	73
2,3-Dimethoxy-1,4-benzoquinone	4	0
2,3-Dimethoxy-5-methyl-1,4-benzoquinone	4	0
2,5-Dihydroxy-1,4-benzoquinone	2	0
2-Methyl-1,4-hydroquinone	8	0
Control	26	0

and 358421/1 1296); 2-methyl-1,4-benzoquinone (toluquinone), >98% (Fluka, Buchs, Switzerland, 89590; lot 298563/1 795 and 358564/1 297); 2,3-dimethyl-1, 4-benzoquinone (synthesized, procedure see below); 2-methoxy-3-methyl-1,4-benzoquinone (synthesized, procedure see below); 2,3-dimethoxy-5-methyl-1,4-benzoquinone, >99% (Fluka, Buchs, Switzerland, 38775; lot 256800/896); 2-methyl-1,4-hydroquinone (toluhydroquinone), >98% (Fluka, Buchs, Switzerland, 89600; lot 339925/1 595); 2,5-dihydroxy-1,4-benzoquinone, 98% (Sigma–Aldrich, Steinheim, Germany, 19,546-4; lot 10643-077). As the dispenser, we used white unperfumed toilet paper of Ivorian production. In the control traps, toilet paper without quinones but, whenever appropriate, with ethanol was exposed.

Synthesis of 2-Methoxy-3-Methyl-1,4-Benzoquinone. The benzoquinone was synthesized after Godfrey et al. (1974) and Luly and Rapoport (1981). The Vilsmeyer complex for a Vilsmeyer–Haack reaction was made by adding 82.5 ml phosphoryl chloride slowly to 150 ml dry *N,N*-dimethylformamide at 0°C. The complex was then added slowly to a solution of 93.1 g of 2,6-dimethyltoluene in 150 ml *N,N*-dimethylformamide at 110°C and was stirred at 110°C for 2 hr. The reaction mixture was diluted with aqueous sodium carbonate and extracted with ethyl acetate. After washing and drying of the organic layer the crude product was purified by crystallization. 2,4-Dimethoxy-3-methylbenzaldehyde (13.5 g) and *m*-chloroperbenzoic acid (25.3 g) were heated under reflux in 400 ml methylene chloride for 24 hr. ^{1}H NMR ($CDCl_3$) δ 1.95 (s, 3H), 4.02 (s, 3H), 6.61 (d, $J = 9$, 1H), 6.68 (d, $J = 9$, 1H).

Synthesis of 2,3-Dimethyl-1,4-Benzoquinone. The benzoquinone was synthesized as described by Sharma et al. (1985). 2,3,4-Trimethoxybenzaldehyde (10 g) and *m*-chloroperbenzoic acid (22.5 g) in 175 ml methylene chloride was stirred for 2 hr at room temperature. One hundred milliliters of 10% sodium thiosulfate solution were added, and stirring was continued for a further 45 min. The mixture was extracted with methylene chloride, washed, and dried. Solvent was removed under vacuum and 50 ml of a mixture of THF and methanol (1:1) were added at 0°C. Ice-cold 10% KOH (25 ml) was added in two portions while shaking. After 15 min, the solution was acidified with 5% HCl, extracted with methylene chloride, washed, and dried. After evaporation of the solvent, the crude oil was dissolved in 250 ml acetonitrile and was added slowly to a solution of ceric ammonium nitrate (25 g) in 250 ml water. Water (500 ml) was added and the mixture was shaken for 5 min. The mixture was extracted with methylene chloride, washed, and dried. The residue was purified by column chromatography and gave orange colored crystals (1.8 g). ^{1}H NMR ($CDCl_3$) δ 4.00 (s, 6H), 6.60 (s, 2H).

Chemical Analysis of the Defensive Secretions. We irritated living millipedes (*Pelmatojulus tigrinus*) mechanically till droplets of defensive secretion were visible on the cuticle, took the substance with filter paper, and extracted it immediately in methylene chloride (chromatography grade). Capillary gas chromatography–mass spectrometry (GC–MS) was performed on a Carlo-Erba HRGC 5160 gas chromatograph (now Fisons, Engelsbach, Germany) coupled with a Varian CH7 mass spectrometer (Varian, Palo Alto, CA). The GC was equipped with a DB-1 fused silica capillary column (30 m $\times$ 0.25 mm ID; df = 0.25 μm; J & W, Folsom, CA) using the following temperature program: 50°C for 2 min, increased at a rate of 20°C/min to 70°C, then 6°C/min to 200°C, and 20°C/min to 300°C, and held for 5 min at 300°C. Injection of 1 μl was carried out in the splitless mode for 1 min with an injection temperature of 300°C and a helium flow rate of 2 ml/min. Electron impact mass spectra (EI-MS) were recorded with an ionization voltage of 70 eV.

Experiments. (a) Natural defensive secretions (Figure 1): During the periods of April 4–May 8, 1996, June 10–August 8, 1997, and April 1–7, 1998, 57 traps baited with defensive secretions of *Pelmatojulus tigrinus* and 33 control traps were exposed before dusk (between 16:30 and 18:00) and collected between 21:00 and 23:00. The flight activity peak of the target species is between 18:00 and 19:00 (personal observations; for *O. latigibber*, see Krell et al., 1998). Our 1996 experiments (included in the present evaluation) have already been described by Krell et al. (1997).

(b) Artificial defensive secretions (Table 1): During the periods of March 20–April 11, 1996, May 6–August 9, 1997, January 31–February 1, 1998, and March 31–April 17, 1998, 84 traps with different quinones and mixtures of quinones (soaked in toilet paper) and 26 control traps (with untreated toilet paper) were

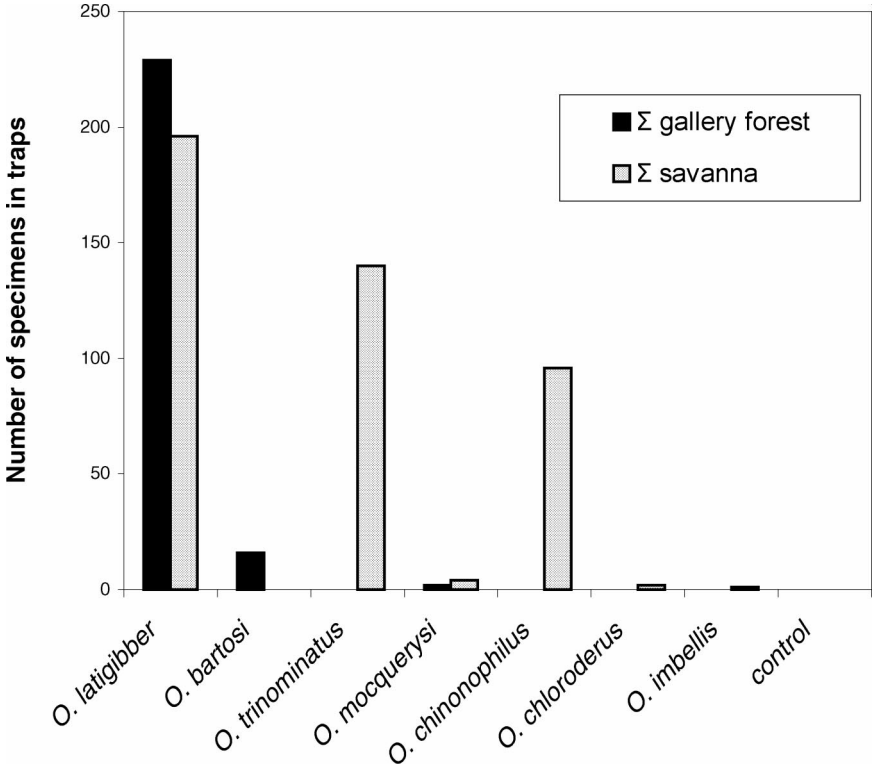

FIG. 1. Numbers of individuals of *Onthophagus* species captured by pitfall traps baited with defensive secretions of *Pelmatojulus tigrinus* (Diplopoda: Spirobolida).

exposed before dusk (18:00, some between 16:00 and 18:00) in the Comoé gallery forest and collected between 20:00 and 22:00.

Beetles. Beetles were identified by F.-T.K. and specimens are deposited in The Natural History Museum, London.

RESULTS

Attracting Effect of Diplopod-Defensive Secretions. In 57 traps baited with defensive secretions of the millipede *Pelmatojulus tigrinus*, we found 686 specimens of seven species of the dung beetle genus *Onthophagus*. With the 33 control traps, no *Onthophagus* specimens were caught (Figure 1).

Millipede-defensive secretions act as attractants for these beetles both in the savanna and in the gallery forest. In the savanna parkland, 439 specimens of six species were caught in the baited traps, while in the gallery forest, 247 specimens

belonging to three species (Figure 1) were caught. *Onthophagus bartosi* Balthasar was only present in the gallery forest. *O. trinominatus* Goidanich, *O. chinonophilus* Krell, *O. chloroderus* d'Orbigny, and *O. imbellis* d'Orbigny were present in the savanna parkland. The most abundant species, *O. latigibber* d'Orbigny, was dominant in both habitats. One of the abundant species is new to science and will be described as *O. chinonophilus* in a separate paper.

Chemical Composition of the Defensive Secretions. We found by GC–MS analysis that the defensive secretions of *Pelmatojulus tigrinus* consist mainly of 2-methyl-1,4-benzoquinone (toluquinone) (EI-MS (70 eV): *m*/*z* (%) 39 (53), 54 (77), 66 (71), 82 (81), 94 (93), 122 (100)) and 2-methoxy-3-methyl-1,4-benzoquinone (EI-MS (70 eV): *m*/*z* (%) 39 (21), 53 (67), 66 (65), 82 (48), 94 (12), 109 (53), 122 (79), 137 (11), 152 (100)). Consequently, we tested these and related substances for attraction of beetles. The proportion of the two components differed between individuals, but 2-methyl-1,4-benzoquinone always occurred in higher concentration.

As minor components, we identified 2-hydroxy-3-methyl-1,4-benzoquinone (EI-MS (70 eV): *m*/*z* (%) 39 (8), 55 (25), 82 (35), 110 (10), 124 (29), 138 (100)), 2-methylhydroquinone (EI-MS (70 eV): *m*/*z* (%) 39 (8), 55 (6), 67 (11), 77 (10), 95 (20), 107 (11), 124 (100)), and 2,4-dihydroxy-6-methylbenzaldehyde (EI-MS (70 eV): *m*/*z* (%) 40 (30), 53 (20), 65 (10), 81 (18), 92 (17), 109 (12), 121 (11), 138 (100), 152 (72)). These components were not present in every sample and may be degradation products of the unstable 2-methoxy-3-methyl-1,4-benzoquinone rather than synthesized by the millipedes.

Attracting Effect of Quinones. In our comparison of the attraction of different quinones on *Onthophagus* dung beetles in the gallery forest, the mixture of the two main components of the defensive secretion (2-methoxy-3-methyl-1,4-benzoquinone and 2-methyl-1,4-benzoquinone) was most successful (Table 1). This mixture attracted 73 *O. latigibber* and 2 *O. bartosi*. The individual components attracted only singletons (2-methyl-1,4-benzoquinone: 2 *O. cyanochlorus* d'Orbigny; 2-methoxy-3-methyl-1,4-benzoquinone: 2 *O. latigibber*, 1 *O. bartosi*, and 1 *O. trinominatus*), whereas the other tested quinones did not attract a single beetle. Hence, the beetles were attracted specifically by the mixture of the two main quinonoid components of the defensive secretions.

DISCUSSION

Repellents as Attractants. Exploitation of olfactory signals, e.g., pheromones, by predators is a common phenomenon (Stowe et al., 1995; Zuk and Kolluru, 1998). However, defensive secretions of arthropods rarely have an attractive effect on other organisms. Single cases are known in which specialized predators use the defensive secretions of their prey to find them. Köpf et al. (1997) found a syrphid fly (Diptera: Syrphidae) to be attracted by the generally noxious salicyl aldehyde

produced by its prey, a leaf beetle larva (Coleoptera: Chrysomelidae). Crab spiders (Araneae: Thomisidae) were attracted by (*E*)-2-octenal and (*E*)-2-decenal, which are components of the defensive secretions of true bugs (Heteroptera) (Aldrich and Barros, 1995). Attraction of *Onthophagus* species to freshly dead millipedes is the first reported case of necrophages being attracted to a resource by its repellents.

Diplopod-defensive secretions did attract *Onthophagus* dung beetles effectively as we have already stated (Krell et al., 1997). It is highly improbable that a considerable number of beetles went into the baited traps by chance, since dung beetles generally do not walk randomly on the ground and do not land in numbers at a place where no resource is present.

Millipede Carcasses as Resource. Quinonoid-defensive secretions are effective repellents to vertebrates and invertebrates (Eisner et al., 1978; Hopkin and Read, 1992), but they do not protect the millipedes perfectly against predators. Beetles, assassin-bugs, ants, scorpions, centipedes, spiders, dormice, shrews, hedgehogs, mongooses, frogs and toads, lizards, turtles, birds, and monkeys are recorded to prey upon quinone-producing diplopods (Lévieux, 1972; Baker, 1985; Lawrence, 1987; Hopkin and Read, 1992; Valderrama et al., 2000; Mahsberg, personal communication). Wedge-capped Capuchin monkeys (*Cebus olivaceus* Schomburgk) anoint themselves with millipedes presumably using the quinonoid-defensive secretion as repellent for flies and mosquitoes (Valderrama et al., 2000; Weldon et al., 2003). Many animals may kill millipedes accidentally by trampling or deliberately as potential food, but neglect them afterwards because of the repellent smell. Phorid flies parasitize juliform millipedes (Disney, 1994). These facts result in fresh millipede carcasses or unused parts of them being available for exploitation by necrophages. If large juliform millipedes are abundant, their carcasses are a regularly available resource for necrophages. However, they contain quinones in high concentrations (Demange, 1993; Huth, 2000) and are, therefore, an unattractive and unsuitable resource for most necrophages.

2-Methyl-1,4-benzoquinone (toluquinone) and 2-methoxy-3-methyl-1,4-benzoquinone are the most common compounds in defensive secretions of juliform Diplopoda (Eisner et al., 1978; Blum, 1981; Attygalle et al., 1993; Huth, 2000). The seven dung beetle species we found in our baited pitfall traps are not only able to cope with the deterrent and toxic quinones, but to use the most common of them as a cue in resource-location. This mechanism enables dung beetles to be recruited to cadavers more rapidly than competitors that use the scent of decomposition when locating them because the quinonoid smell emerges immediately when a millipede is killed, whereas the smell of rotting appears later.

Since large juliform millipedes are abundant in the African tropics (Dangerfield, 1990; Mahsberg, 1997) specialization on their fresh carcasses, which are unattractive for most necrophagous competitors, is probably an advantageous strategy. Indeed most of the individuals found in our traps belong to species specialized on millipede carcasses (*O. latigibber*, *O. bartosi*, *O. trinominatus*,

O. chinonophilus, *O. chloroderus*, and *O. imbellis*; Cambefort, 1984; personal observation). As with most "specialized" dung beetle species, this means that they strongly prefer the particular resource, but are occasionally found on other resources. Singletons of *O. latigibber* have been found on reptile and amphibian carrion and on human feces and buffalo dung (Cambefort, 1984; personal observation). *O. bartosi* was caught in traps with fish and amphibian carrion by Cambefort (1984). *O. mocquerysi*, which was only occasionally attracted to defensive secretions (Table 1), and *O. cyanochlorus* d'Orbigny, which was found once in a trap with toluquinone, seem to be generalists. Cambefort (1984) considered the former to be a satellite species in coprocenoses of human feces and other "petits excréments les plus divers," and it was even found in elephant dung (Frey, 1961). However, 99.0% (758) of the 767 specimens captured in all experiments belonged to specialist species.

High abundance and permanent availability of a resource during the activity period of potential users may cause specialization of the users involving complex adaptations (i.e., coping with the toxicity of quinones). Are there users of dead millipedes if this resource is rare?

Attraction of dung beetles by quinonoid-defensive secretions of diplopods is not limited to the Afrotropical region. In Borneo, where large millipedes are rare, we found *Onthophagus penicillatus* Harold, *O. rudis* Sharp, and two species of Hybosoridae (Scarabaeoidea) attracted by the quinonoid-defensive secretions of Harpagophoridae (Brühl and Krell, 2003). All of these beetle species are generalist necrophages. At least for *O. penicillatus*, necrophagy on juliform millipedes is known (Masumoto, 2001). Although the resource is rare, these beetles are adapted on and even attracted by the quinonoid-defensive secretions. However, since the resource is rare, specialization was obviously not an advantageous strategy.

Attractants for Dung Beetles and Other Scarabaeoidea. Odorous components of feces that attract coprophagous dung beetles (including *Onthophagus* species) most efficiently, particularly as a bouquet, are 2-butanone, cresol, indole or skatole, and butyric acid (unpublished results; Inouchi et al., 1988). Phenol is also attractive (Inouchi et al., 1988). It is conceivable that receptors for cyclic compounds might have been modified in evolution to become sensitive to benzoquinones, since, for instance, phenol and cresol are structurally closely related to benzoquinones. Recently, benzoquinones were found to attract phytophagous and xylophagous Scarabaeidae of other clades: 1,4-benzoquinone is a sexual pheromone of a European cockchafer (*Melolontha hippocastani* Fabricius, Melolonthinae; Ruther et al., 2001) and an attractant for males of the chafer *Rhizotrogus vernus* Germar (Melolonthinae; Imrei et al., 2002). The same substance and different substituted benzoquinones, among them toluquinone and 2-methoxy-3-methyl-1,4-benzoquinone, attracted an Afrotropical rhinoceros beetle (Dynastinae; Krell et al., 1999). Because of the possible modification of receptors to be perceptive for quinones and the existence of quinone receptors in other clades of the Scarabaeidae,

the exploitation of a novel resource using quinones as odorous cues was probably only a small evolutionary step for the *Onthophagus* species.

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OLFACTORY RESPONSES TO APHID AND HOST PLANT VOLATILE RELEASES: (*E*)-β-FARNESENE AN EFFECTIVE KAIROMONE FOR THE PREDATOR *Adalia bipunctata*

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Abstract—The volatiles released from several aphid and host plant species, alone or associated, were studied for their infochemical role in prey location. Using a four-arm olfactometer, the attraction of several combinations of three aphid (*Myzus persicae*, *Acyrthosiphon pisum*, and *Brevicoryne brassicae*) and three plant (*Vicia faba*, *Brassica napus*, and *Sinapis alba*) species toward *Adalia bipunctata* larvae and adults was observed. Both predatory larvae and adults were attracted only by *A. pisum* and *M. persicae* when they were crushed, whatever the host plant. (*E*)-β-Farnesene, the aphid alarm pheromone, was the effective kairomone for the ladybird. Plant leaves alone (*V. faba*, *B. napus*, and *S. alba*) or in association with nonstressed whole aphids (the three species) did not have any attraction for the predator. The *B. brassicae* specialist aphid is the only prey that was not attracted to *A. bipunctata* larvae and adults, even if they were crushed. Release of *B. brassicae* molecules similar to the host plant allelochemicals was demonstrated by GC–MS analysis. The lack of behavioral response of the ladybird at short distance toward the cruciferous specialist aphid was related only to the absence of (*E*)-β-farnesene in the aphid prey volatile pattern.

Key Words—Infochemical, predator, prey localization, olfactometer, β-farnesene, kairomone.

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INTRODUCTION

Predators and parasitoids, with their complex biology, elaborate interactions with other organisms, and importance in pest control, are fascinating subjects for ecological studies. Beneficial insects are sensitive to chemical aspects of the multitrophic environment, particularly with regard to host location (Poppy, 1997). To localize prey in natural habitats, entomophagous insects use numerous chemical cues emitted by prey and host plants, alone or in association (Vet and Dicke, 1992). Many different chemical cues correspond to a diversity of associations between potential prey and host plant species. However, little is known about chemical communication among predatory insects. Recently, reports of electroantennogram (EAG) recordings from three predatory insect species, namely *Coleomegilla maculata* (Coleoptera, Coccinellidae), *Chrysoperla carnea* (Nevroptera, Chrysopidae; Zhu et al., 1999), and *Coccinella septempunctata* (Coleoptera, Coccinellidae; Al Abassi et al., 2000) showed significant EAG responses to semiochemicals released from potential prey and host plants. These predators possibly use such chemicals to locate their prey. Ninkovic et al. (2001) also demonstrated that the seven-spotted ladybird, *C. septempunctata*, responded positively to volatiles from the aphid, *Rhopalosiphum padi*, and to infested plants of *Hordeum vulgare*. Two molecules, namely (*E*)-β-farnesene and β-caryophyllene, were found to be a kairomone and an informative inhibitor, respectively, for the seven-spot ladybird by electroantennography and olfactometry methods (Al Abassi et al., 2000).

To study the relation between volatile emissions from aphids and host plant complexes, the foraging behavior of the predator and localization of the aphid prey by the predatory ladybird was studied using a four-way olfactometer. This work was designed to observe the predator response toward chemical cues emitted by the host plant, the aphid (nonstressed whole insect or crushed), alone or in combination. Different odor sources, corresponding to potential situations met by the ladybird in its natural habitat were tested. In parallel, GC–MS analyses of the tested odor source samples were performed to identify the volatile compounds affecting the predator behavior. The relationship between prey suitability, volatile release, and behavioral response of the predator are discussed in relation to the potential use of infochemicals in the biological control of aphids.

METHODS AND MATERIALS

Plant and Insect Rearing. Broad beans (*Vicia faba* L.), white mustard (*Sinapis alba* L.), and oilseed rape (*Brassica napus* L.) were grown in 10 cm diam. plastic pots in three separate controlled environmental rooms at 20 $\pm$ 2°C and under a 16/8 hr L/D photoperiod. While beans were cultivated in pots containing a 1:1 mixture of perlite:vermiculite, Brassicaceae species were sown in 20 $\times$ 30 cm plastic trays containing ordinary compost, and were transplanted into plastic pots with the same compost when the plants had two true leaves.

Acyrthosiphon pisum. (Harris) and *Myzus persicae* Sultzer were reared on the three host plant species, while *Brevicoryne brassicae* L. was reared only on crucifer species. Plants were inoculated at the 5–6 true leaf stage with one of the aphid species. Each combination of aphid and plant was isolated in separated conditioned rooms at 20 ± 2°C and under a 16/8 hr photoperiod. Mass rearing of *Adalia bipunctata* L. was maintained for many years in the laboratory. Both control adults and larvae were reared in aerated plastic boxes and fed with *A. pisum* on *V. faba*. From hatching, larvae that were used in olfactometry assays were individually reared in 5 cm diam. Petri dishes. Ladybirds (72-hr-old) were used to test the chemical cues at the larval stage. Other beetle larvae achieved the adult stage in Petri dishes. One-week-old adults were used to observe the infochemical role of the various tested odor sources. Ladybirds were fed with an excess of *A. pisum* reared on *V. faba* in individual Petri dishes.

Olfactometer. The four-way olfactometer that was used to test the behavioral responses of second instars and adult ladybirds toward several stimuli was similar to the one described by Vet et al. (1983). Compressed air was circulated through active charcoal and a water bottle before entering the exposure chamber. Air left the latter through a hole in the chamber roof. Airflow in each of the four arms was adjusted with a flow meter to 60 ml min^{-1}, thereby creating four equal distinct fields in the chamber. Odor emitting samples were placed into a 25-ml airtight glass flask linked by plastic tube to one of the four olfactomer arms. The olfactometer system was placed into a controlled temperature room at 20 ± 2°C. Before the beginning of the assays, the system was cleaned with pure ethanol and rinsed with distilled water.

Odor Sources as Chemical Cues. Several stimuli were tested as odor sources:

1. Undamaged, nonstressed whole aphids (*A. pisum*, *M. persicae*, or *B. brassicae*): Aphid samples (250 mg) previously collected from one of the host plants were carefully placed into 25-ml glass flasks.
2. Crushed aphids: one of the three species (250 mg) having an odor source similar to a stressed aphid colony. Aphid samples were rapidly crushed with glass rods in 25-ml glass flasks and immediately covered with glass tops to be airtight.
3. Host plant sections (*V. faba*, *B. napus*, or *S. alba*): Stems (5 cm) with three leaves were cut from healthy uninfested plants and placed into glass flasks.
4. Aphid and host plant sections: combinations of the three plant and three aphid species having volatile production that resulted in aphid feeding on plants. Five-centimeter stems infested with aphids (250 mg) were placed into glass flasks.

Aphid and/or host plant samples in glass flasks were placed into the air stream of one of the four arms of the olfactometer. Complementary assays using pure (*E*)-β-farnesene, a well-known aphid alarm pheromone as odor source, were performed. The latter molecule was purified from *A. pisum* lipids using microcolumn

chromatography (40 × 5 mm, 70–230 mesh silica gel E60 column) with 2-hexane as eluent. The observation method for the use of (*E*)-β-farnesene was similar to the one described to test aphid or plant samples as chemical cues. (*E*)-β-Farnesene was injected into the glass flask using a Hamilton syringe. We waited 3 min before joining the odor source to the exposure chamber of the olfactometer to allow for evaporation of hexane prior to starting observations.

Behavioral Observations. Second instars (72-hr-old) and adults ($N = 20$ per stimulus) were individually observed for 20 min in the olfactometer. The olfactometer was divided into one central area and four others related to the four odor sources. Ladybird durations in each area were determined. Insect localization in one of the four areas at the end of the observation was considered the final behavioral choice of the predatory ladybird. After every five observations, the position of the odor fields was changed. The exposure chamber was cleaned with pure ethanol and rinsed with distilled water after each assay.

Analysis of Volatile Releases from Aphids and Plants. Aphid and plant samples (250 mg) were crushed with a glass pestle in a glass tube adapted to the SPME method. Each aphid species was tested at least in duplicate. Crushed samples were first maintained for 30 min at 30.0 ± 0.2°C in glass tubes adapted to SPME. The volatile metabolites were sampled for 30 min with 100-μm PDMS (polydimethylsiloxane) SPME fibers from Supelco® and immediately analyzed by GC–MS on an Hewlett-Packard HP5972 mass spectrometer coupled with an HP5890 series II gas chromatograph. The following analytical conditions were used: split–splitless injection at 250°C, HP5-MS (5% phenyl-dimethylpolysiloxane) column (30 m × 0.25 mm, df = 1 μm). Samples were purged with He at 4 ml min^{-1} for 11 min, and the temperature program was from 40°C (1 min hold) to 180°C at 6°C min^{-1} then to 280°C at 15°C min^{-1}. MS spectra were obtained in the EI mode at 70 eV (scanned mass range from 30 to 300 amu). The analytes were identified on the basis of their retention times and by interpretation of MS fragmentation patterns. Spectra were compared to those of the Wiley238.L spectral library. (E)-β-Farnesene in hexane was analyzed by the same procedure.

Statistical Analysis. Observed frequencies related to the final choice of *A. bipunctata* in localizing prey were compared to corresponding theorical frequencies (one odor source and three controls) using a χ^2 test. Relative stay durations of ladybirds were compared by the contrast method using the residual mean square from ANOVA after arcsin$\sqrt{x}$ transformation (Dagnelie, 1973).

RESULTS

GC–MS chromatograms of volatiles from aphids and host plants are presented in Figure 1. Volatile compounds were not detected from nonstressed whole aphids of the three tested species. Only crushed *A. pisum* and *M. persicae* released (*E*)-β-farnesene whatever the host plant species. Molecules similar to host

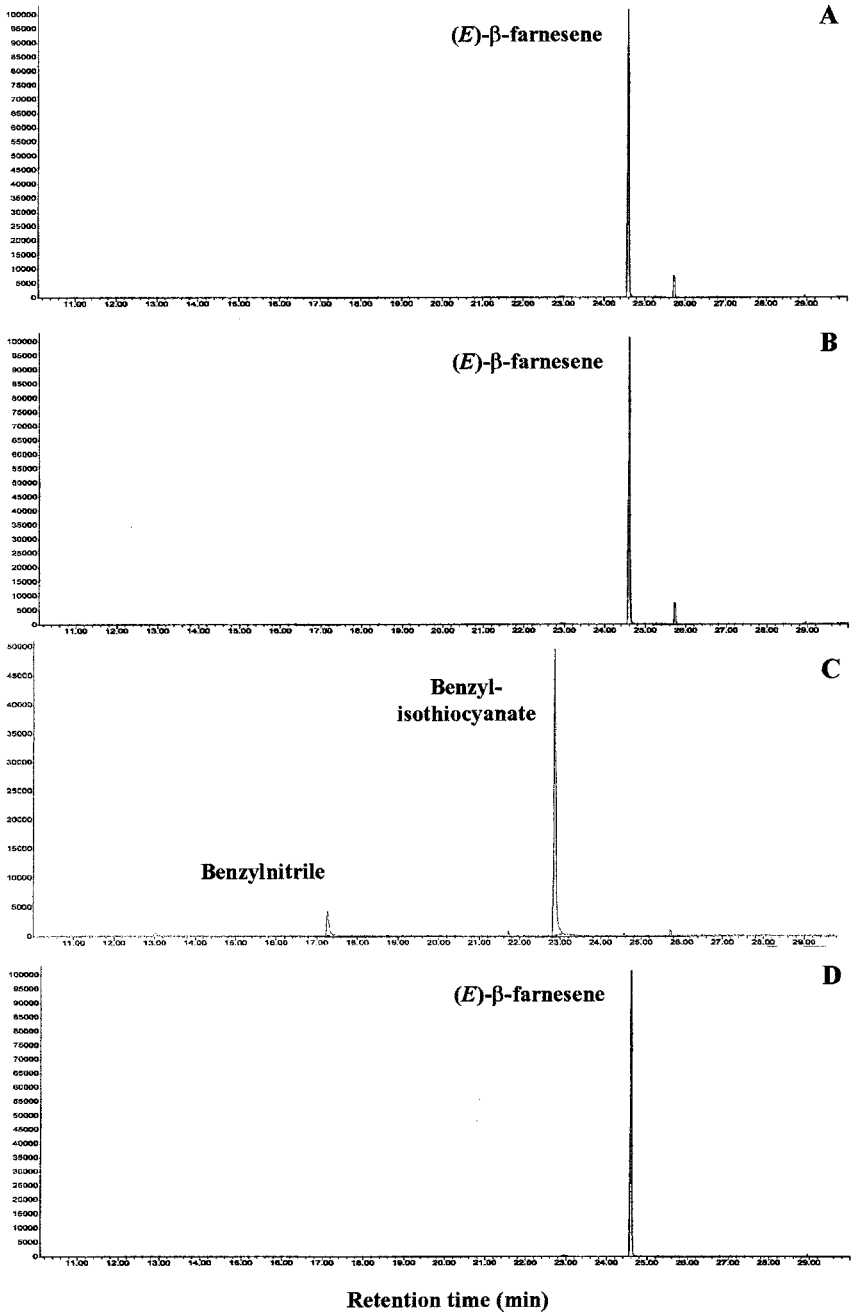

FIG. 1. GC–MS chromatograms of volatile releases from *Myzus persicae* (A) and *Acyrthosiphon pisum* (B) both reared on *Vicia faba*, *Brevicoryne brassicae* reared on *Sinapis alba* (C). The chromatogram of the (*E*)-β-farnesene purified solution in hexane is also presented (D).

plant allelochemicals, namely benzyl-isothiocyanate and the benzylnitrile, were found when crushed *B. brassicae* reared on *S. alba* was analyzed. Volatiles from *B. brassicae* reared on *B. napus* were not detected. This was linked to the low concentration of glucosinolate precursors in the aphid host plant. Both Brassicaceae plant species emitted degradation products of glucosinolates (nitriles and isothiocyanates); however, volatile were not detected from broad bean samples.

Prey localization by the ladybird at both adult and larval stages using crushed or nonstressed whole aphids reared on three different host plants are presented in Table 1. Two aphids, *A. pisum* and *M. persicae*, attracted the predatory ladybirds at

TABLE 1. RESPONSES OF *Adalia bipunctata* ADULTS (A) AND SECOND INSTARS (B) TOWARD CRUSHED AND NONSTRESSED WHOLE APHIDS REARED ON DIFFERENT HOST PLANT SPECIES[a]

Host plant and aphid combinations			Observed frequencies	χ^2	P
(A)					
Vicia faba	*A. pisum*	Whole insects	0.4	2.40	0.121
		Crushed	0.45	4.27	0.039*
	M. persicae	Whole insects	0.35	1.07	0.301
		Crushed	0.50	6.67	0.010**
Brassica napus	*M. persicae*	Whole insects	0.40	2.40	0.121
		Crushed	0.45	4.27	0.039*
	B. brassicae	Whole insects	0.30	0.27	0.603
		Crushed	0.25	0.00	1.000
Sinapis alba	*M. persicae*	Whole insects	0.35	1.07	0.301
		Crushed	0.45	4.27	0.039*
	B. brassicae	Whole insects	0.20	0.27	0.603
		Crushed	0.30	0.27	0.603
(B)					
Vicia faba	*A. pisum*	Whole insects	0.40	2.40	0.121
		Crushed	0.50	6.67	0.010**
	M. persicae	Whole insects	0.30	0.27	0.603
		Crushed	0.45	4.27	0.039*
Brassica napus	*M. persicae*	Whole insects	0.30	0.27	0.603
		Crushed	0.50	6.67	0.010**
	B. brassicae	Whole insects	0.15	1.07	0.301
		Crushed	0.25	0.00	1.000
Sinapis alba	*M. persicae*	Whole insects	0.30	0.27	0.603
		Crushed	0.45	4.27	0.038*
	B. brassicae	Whole insects	0.25	0.00	1.000
		Crushed	0.20	0.27	0.603

[a] Observed frequencies related to the final choice of *A. bipunctata* ($N = 20$) to localize the odor sources were compared to the corresponding theorical frequency (one odor source and three controls) using a χ^2 test.

* and ** indicate significant differneces at $P < 0.05$ and $P < 0.01$, respectively.

larval and adult stages when the aphids were crushed regardless of their host plants. No significant effect was observed when the nonstressed whole aphids reared on one of the tested host plants was used as an odor source.

Plant species, alone or in association with aphids, were also tested as chemical cues. When leaves of *V. faba*, *B. napus*, or *S. alba* alone were used as odor sources, no significant attraction was observed on the final choice of the ladybird larvae ($\chi^2 = 0.27$ and $P = 0.603$, $\chi^2 = 0.27$ and $P = 0.603$, $\chi^2 = 1.07$ and $P = 0.301$) and adults ($\chi^2 = 0.27$ and $P = 0.603$, $\chi^2 = 1.07$ and $P = 0.301$, $\chi^2 = 2.40$ and $P = 0.121$).

Associations of plant leaves and nonstressed whole aphids were used without having any significant informative effect as semiochemicals on prey localization by the ladybird. *A. pisum* or *M. persicae* on *V. faba* ($\chi^2 = 0.27$ and $P = 0.603$, $\chi^2 = 0.00$ and $P = 1.000$), *M. persicae* or *B. brassicae* on *B. napus* ($\chi^2 = 1.07$ and $P = 0.301$ twice) and on *S. alba* ($\chi^2 = 1.07$ and $P = 0.301$, $\chi^2 = 0.27$ and $P = 0.603$) did not attract predatory beetle larvae. Adults of *A. bipunctata* did not respond to molecules released from nonstressed whole aphid and host plant combinations: *A. pisum* or *M. persicae* on *V. faba* ($\chi^2 = 1.07$ and $P = 0.301$, $\chi^2 = 0.27$ and $P = 0.603$), *M. persicae* or *B. brassicae* on *B. napus* ($\chi^2 = 1.07$ and $P = 0.301$, $\chi^2 = 0.27$ and $P = 0.603$) and on *S. alba* ($\chi^2 = 2.40$ and $P = 0.121$, $\chi^2 = 1.06$ and $P = 0.301$).

Observation of times spent by the ladybirds in each of the four olfactometer arms, corresponding to the four air arrivals including the tested odor source, confirmed the attractive effect of crushed *A. pisum* and *M. persicae* reared on *V. faba* (Figure 2), on *B. napus* (Figure 3), and on *S. alba* (Figure 4) for adults of *A. bipunctata*. Similar results were observed when testing the former aphid and host plant combinations on the two spot ladybird larvae (Table 2). Nonstressed whole aphids from *A. pisum* and *M. persicae* were not attractive for predator larvae or adults. The third prey species, *B. brassicae*, never attracted the predatory ladybirds even if the insect odor samples were crushed (Figure 4). Associations of nonstressed whole aphids and plant leaves, or the latter alone, were also used as chemical cues. When *V. faba*, *B. napus*, or *S. alba* leaves were tested as odor sources, attraction of ladybird larvae was not observed on the times spent in the olfactometer field related to the odor source samples ($F = 0.23$ and $P = 0.632$, $F = 0.26$ and $P = 0.578$, $F = 0.29$ and $P = 0.531$).

Adults of ladybirds were not attracted by the volatiles released from the three plant species when used alone ($F = 0.28$ and $P = 0.404$, $F = 1.87$ and $P = 0.825$, $F = 1.40$ and $P = 0.321$ for *V. faba*, *B. napus*, and *S. alba*, respectively). Association of leaves and nonstressed whole aphids were also tested, but did not present any significant effect on the duration ladybirds stayed in the olfactometer area corresponding to the odor source. *A. pisum* or *M. persicae* on *V. faba* ($F = 1.08$ and $P = 0.302$, $F = 1.43$ and $P = 0.289$), *M. persicae* or *B. brassicae* on *B. napus* ($F = 1.22$ and $P = 0.728$, $F = 1.45$ and $P = 0.768$), and on *S. alba*

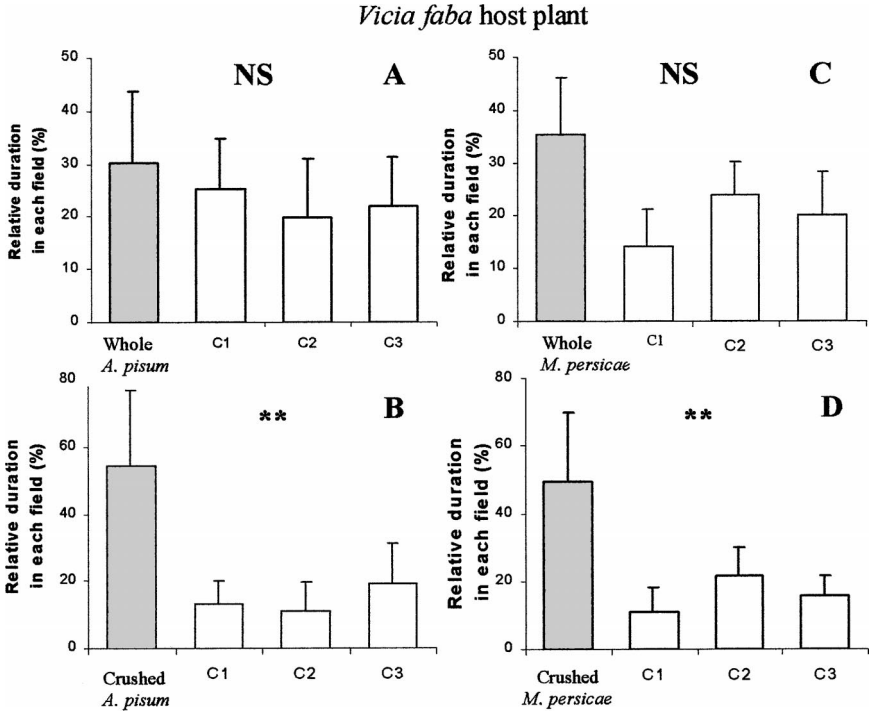

FIG. 2. Mean relative time (%) spent in each field of a four-arm olfactometer by *Adalia bipunctata* adults exposed to nonstressed whole or crushed *Acyrthosiphon pisum* (A and B, respectively) or *Myzus persicae* (C and D, respectively) both reared on *Vicia faba*. C1, C2, and C3 are the control air sources related to three of the four olfactometer arms. Error bars represent standard deviation of the mean. NS and ** indicate no significance and significant differences at $P < 0.01$, respectively.

($F = 0.25$ and $P = 0.382$, $F = 0.30$ and $P = 0.412$) did not attract predatory beetle larvae.

Molecules released by the plant and nonstressed whole aphid combinations did not have any significant effect on the time spent by *A. bipunctata* adults in the olfactometer exposure chamber. *A. pisum* or *M. persicae* on *V. faba* ($F = 0.97$ and $P = 0.408$, $F = 1.27$ and $P = 0.309$), *M. persicae* or *B. brassicae* on *B. napus* ($F = 1.53$ and $P = 0.215$, $F = 1.04$ and $P = 0.311$), and on *S. alba* ($F = 1.04$ and $P = 0.311$, $F = 0.21$ and $P = 0.644$) did not induce any significant difference of time spent by the predator adults in the field related to the tested odor source.

A hexane solution of natural (E)-β-farnesene purified from *A. pisum* only includes this molecule (Figure 1). Before beginning the olfactometry assays with the aphid alarm pheromone, a hexane control was used. The foraging behavior of the ladybird was not affected by hexane as a chemical cue ($0.27 < \chi^2 < 1.07$ and

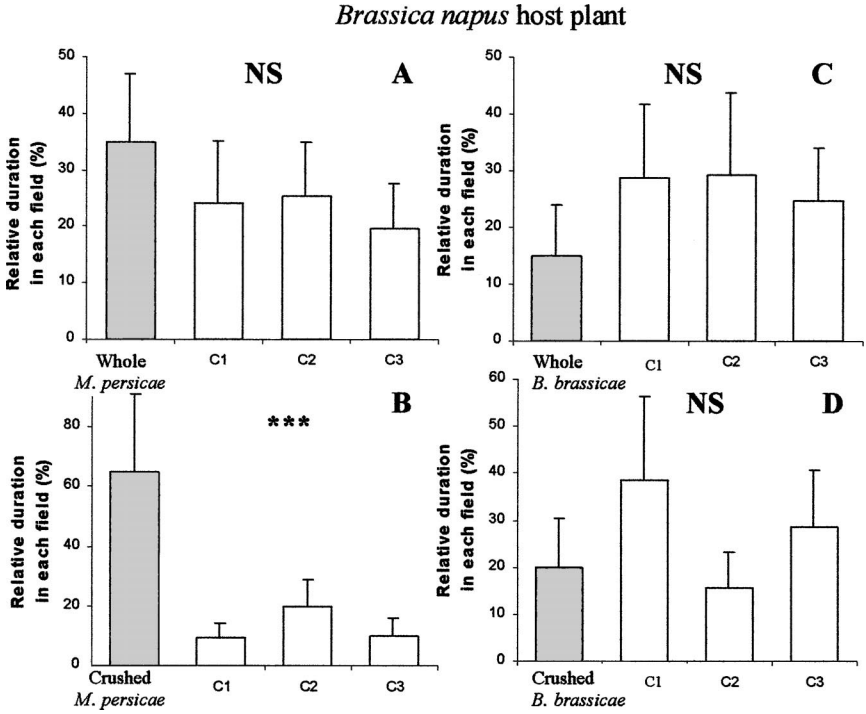

FIG. 3. Mean relative time (%) spent in each field of a four-arm olfactometer by *Adalia bipunctata* adults exposed to nonstressed whole or crushed *Myzus persicae* (A and B, respectively) or *Brevicoryne brassicae* (C and D, respectively) both reared on *Brassica napus*. C1, C2, and C3 are the control air sources related to three of the four olfactometer arms. Error bars represent standard deviation of the mean. NS and *** indicate no significance and significant differences at $P < 0.001$, respectively.

$0.603 < P < 0.301$). In contrast, both predatory larvae and adults were attracted by (*E*)-β-farnesene if the solution included more than 2 μg of the informative molecule ($\chi^2 = 6.67$ and $P = 0.010$, $\chi^2 = 8.47$ and $P < 0.006$, respectively). The stay durations of the ladybirds in the olfactometer area related to the aphid alarm pheromone solution was significantly higher than the durations corresponding to the other areas ($F = 11.23$ and $P < 0.001$, $F = 9.89$ and $P = 0.002$ for the larvae and adults, respectively).

DISCUSSION

Comprehension of the chemical ecology of plant–insect relations is a key factor in determining the way entomophagous beneficial insects localize host plants

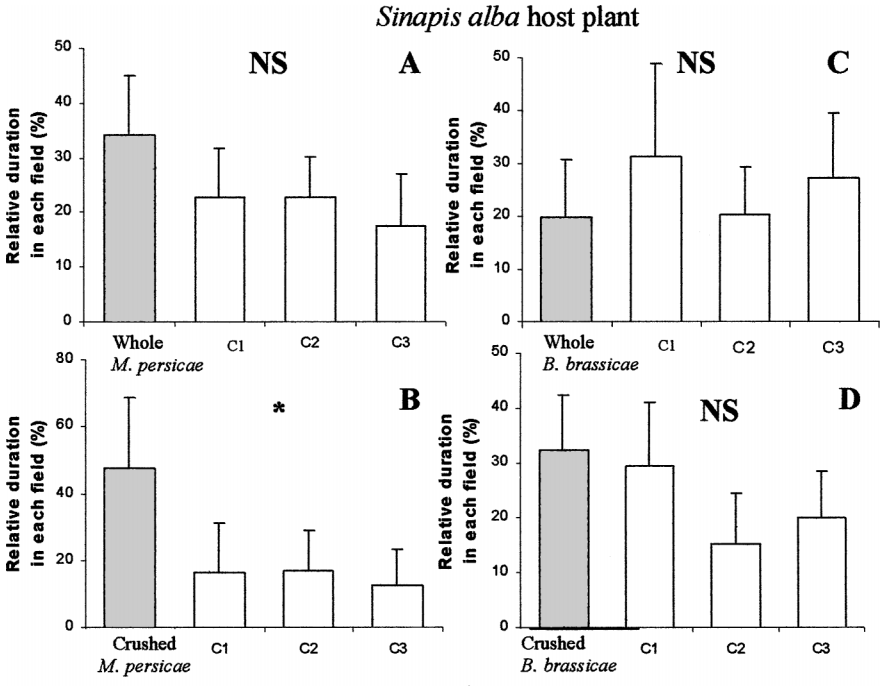

FIG. 4. Mean relative time (%) spent in each field of a four-arm olfactometer by *Adalia bipunctata* adults exposed to nonstressed whole or crushed *Myzus persicae* (A and B, respectively) or *Brevicoryne brassicae* (C and D, respectively) both reared on *Sinapis alba*. C1, C2, and C3 are the control air sources related to three of the four olfactometer arms. Error bars represent standard deviation of the mean. NS and * indicate no significance and significant differences at $P < 0.05$, respectively.

or prey. While the semiochemicals emitted by plants can explain the orientation and distribution of aphids, these substances also play an infochemical role for the aphidophagous natural enemies. In the first steps of prey searching, predators localize prey habitat by using chemical cues emitted by plants (Tumlinson et al., 1992). In our experiments, plant volatiles did not attract either adults or larvae of *A. bipunctata* at short distances. Other examples also illustrate that isolated plants are not universal sources of infochemicals for entomophagous beneficials. Indeed, *Diaeretella rapae*, an aphid parasitoid is not attracted by noninfested Brassicaceae leaves (Reed et al., 1995). Whether *C. septempunctata* responds positively to volatiles from aphids, *R. padi*, from *H. vulgare* infested plants, ladybirds do not react to volatiles from uninfested plants in olfactometer bioassays (Ninkovic et al., 2001). Neveu et al. (2002) also demonstrated that the parasitoid, *Trybliographa rapae* (Hymenoptera: Figitidae) is not attracted to volatiles emanating

TABLE 2. RELATIVE DURATION (%) SPENT BY *Adalia bipunctata* SECOND INSTARS IN THE OLFACTOMETER ARM CORRESPONDING TO THE TESTED ODOR SOURCE (CRUSHED OR NONSTRESSED) WHOLE APHIDS REARED ON DIFFERENT HOST PLANT SPEICES[a]

Host plant and aphid combinations			Relative duration in odor field (%)	*F*	*P*
Vicia faba	*A. pisum*	Whole insects	30.4 ± 21.5	0.37	0.544
		Crushed	45.4 ± 17.3	4.9	0.030*
	M. persicae	Whole insects	27.9 ± 20.8	0.15	0.704
		Crushed	43.2 ± 16.1	4.75	0.032*
Brassica napus	*M. persicae*	Whole insects	14.1 ± 6.3	1.22	0.272
		Crushed	45.8 ± 20.7	4.15	0.045*
	B. brassicae	Whole insects	13.2 ± 7.7	1.45	0.232
		Crushed	17.9 ± 9.2	0.37	0.544
Sinapis alba	*M. persicae*	Whole insects	29.7 ± 10.6	0.25	0.617
		Crushed	46.1 ± 19.1	4.27	0.039*
	B. brassicae	Whole insects	19.1 ± 7.9	0.30	0.588
		Crushed	28.6 ± 12.2	0.38	0.538

[a] Relative stay durations of ladybrids ($N = 20$) were compared by the contrast method using the residual mean square from ANOVA after $\arcsin\sqrt{x}$ transformation.
* indicates significants differences at $P < 0.05$.

from uninfested turnips either as whole plants, roots, or leaves. The lack of response of both entomophagous predators and parasitoids was attributed to the low reliability of this signal to inform the beneficial species of host preference (Vet and Dicke, 1992).

None of the plant–aphid complexes that were tested was attractive to *A. bipunctata*. These results are in accordance with previous work on beneficials when a too low concentration of volatile liberation by the aphid and host plant samples was used. The foraging behavior of the aphid parasitoid *Aphidius ervi* was influenced by semiochemicals emitted by aphid infested plants when a certain threshold of infestation, in terms of number of aphids and hours of feeding activity, was reached (Guerrieri et al., 1999). A volatile dose-dependent response of another predatory beetle, *C. septempunctata*, toward prey–host plant complex was also observed. Significant differences toward odor source and control in a Y olfactometer were only observed when the seven spot ladybird was exposed to at least 30 aphid damaged shoots with 1200 tea aphids. Below this amount of volatile emitting biological sample, no significant attraction of the predator was observed (Han and Chen, 2002).

Aphid samples alone were tested as potential kairomone cues for *A. bipunctata*. Our results allowed us to demonstrate that there was no systematic response of a polyphagous aphid predator toward volatiles released from several potential

prey. When crushed *M. persicae* and *A. pisum* reared on several host plant species were used, predatory ladybird responded positively to the emitted chemical cues. When whole aphids were used alone, no informative effect was observed. Similar results have been obtained by Du et al. (1996). The parasitoid *A. ervi* was not attracted by whole *A. pisum* aphids. An hypothesis was proposed to explain these observations: crushed aphids release higher levels of volatile substances. The amount of emitted molecules is then sufficient to be perceived by the predators. The chemical cues released by whole aphids were not sufficient to allow prey localization by ladybirds. Volatile molecules were not detected by GC–MS analysis of whole aphid samples in our experiments. Larger amounts of whole aphids had to be used as odor sources to be localized by predators in our olfactometry assays. Using another prey species, Han and Chen (2002) showed that at least 2000 tea aphids were needed to emit enough odor to attract *C. septempunctata*. Moreover, these authors found that *C. septempunctata* was the most sensitive species of three tested natural enemies.

The other aphid species, *B. brassicae*, was not attractive to ladybirds. *B. brassicae* (whole insects or crushed), alone or in association with host plant leaves, did not attract larvae or adults of *A. bipunctata*. In this case, two hypotheses might explain the lack of infochemical effect. Substances emitted by plants and/or the crucifer specialist aphids modified the kairomonal composition to the predatory ladybirds when compared to chemical cues from *M. persicae* and *A. pisum*. Al Abassi et al. (2000) demonstrated that the attractivity of (*E*)-β-farnesene for *C. septempunctata* decreased with increasing amounts of β-caryophyllene. The isothiocyanate emission that was detected by GC–MS from *B. brassicae* could act as a kairomone inhibitor such as the β-caryophyllene that informs the predator of prey unsuitability. For example, volatile isothiocyanates released from cruciferous plants stimulate the olfactory receptors of generalist herbivore insects such as *Aphis fabae*. The pentenyl- and butyl-ITC were repellent to this aphid species (Isaacs et al., 1993). These molecules could also repel generalist predators such as *A. bipunctata*. To confirm the presence of plant secondary substances in *B. brassicae*, analysis of molecules released by the tested aphid and host plant species, each alone or in association, were partially performed in previous work (Francis et al., 2001a) and completed here. The alternative hypothesis is that volatile molecules released by whole or crushed *B. brassicae* do not present any informative effect. As (*E*)-β-farnesene was not the major molecule emitted by the aphid (as from *A. pisum* and *M. persicae*), *B. brassicae* did not produce efficient kairomone for natural enemies, but developed a system of defense similar to its Brassicaceae host plants (Francis et al., 2000a,b, 2001b).

GC–MS analyses allowed us to show the attractive effect of (*E*)-β-farnesene over short distances when emitted alone by *A. pisum* and *M. persicae*, regardless of the host plant they fed upon, when in sufficient concentration. Other volatiles such as some isothiocyanates and nitriles from *B. brassicae* provide different chemical

cues to ladybirds. The beetles do not respond to a mixture of volatiles similar to the ones from Brassica host plants. Although the crucifer specialist *Lipaphis erysimi* responded to a mixture of (*E*)-β-farnesene and isothiocyanate, (*E*)-β-farnesene alone did not act as alarm pheromone (Dawson et al., 1987).

Ladybirds were attracted by (*E*)-β-farnesene as an olfactory cue with a dose-dependent factor. When the amount of (*E*)-β-farnesene was less than 2 μg, a significant attraction was not observed for the coccinellid. Absence of an informational role of whole *M. persicae* and *A. pisum* seemed to be due to the lower liberation of (*E*)-β-farnesene from undamaged aphids. Moreover, the kairomone role of (*E*)-β-farnesene on beneficial insects was not systematic. While the predatory species *A. bipunctata* was attracted by that latter terpene molecule, *Chrysopa cognata*, another aphid predator did not react to it as an olfactory cue (Boo et al., 1998). In contrast, flight assays in tunnels allowed observation of the (*E*)-β-farnesene attractive effect on the parasitoid *A. ervi* (Du et al., 1998). Moreover, prey searching activity of polyphagous predators from the Carabidae family was increased by the monoterpene release from *Sitobion avenae* aphid (Kirkland et al., 1998). Two carabid beetle species also are highly sensitive to (*E*)-β-farnesene: *Pterostichus melanarius* and *Harpalus rufipes* (Kielty et al., 1996).

In summary, this work shows that *A. bipunctata* only reacts to semiochemical cues from prey. Host plant and aphid–host plant complexes did not represent effective infochemical sources at short distances under our experimental conditions. Identification of potential (*E*)-β-farnesene synergists or inhibitors for the two spot ladybird is in progress by studying different ratios of plant volatiles (nitriles, isothiocyanates, and several terpenes) as odor sources. The results of complementary studies using plant–insect volatile releases will allow us to determine the effective infochemicals on predator flights. (*E*)-β-Farnesene, a well-known aphid alarm pheromone, alone was an effective kairomone for the two spot ladybird. The differential response of a polyphagous aphid predator to several potential prey demonstrates that biological control cannot be generalized. Each pest and cultivated plant species must be considered as a unique situation. Nevertheless, (*E*)-β-farnesene might be a promising molecule for use as a biopesticide attractant for several aphidophagous predators including *A. bipunctata*.

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CONSTRAINT OF FEEDING BY CHRONIC INGESTION OF 1,8-CINEOLE IN THE BRUSHTAIL POSSUM (*Trichosurus vulpecula*)

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Abstract—*Eucalyptus* leaf-eating marsupials such as the brushtail possum (*Trichosurus vulpecula*) ingest large amounts of terpenes, especially 1,8-cineole (cineole)—the major component of many eucalyptus oils. Brushtail possums were acclimated to a non-*Eucalyptus* diet with increasing concentrations of cineole (0.5–4.0% wet weight) added over 18 d. We measured food and cineole consumption and urinary metabolites of cineole. Food intake decreased with cineole content, indicating that it was constrained by the maximum tolerable intake of cineole that was 3.8 ± 0.2 g kg^{-1} or 5.2 ± 0.3 g kg$^{-0.75}$ (mean $\pm$ SE, $N = 6$). The pattern of metabolites was similar at all cineole intakes (56% hydroxycineolic acids, 27% cineolic acids, 13% hydroxycineoles, and 4% dihydroxycineoles). In another experiment, possums maintained on artificial diet were abruptly presented with 4% cineole for 5 d. Food intake fell by $45 \pm 6\%$ (mean $\pm$ SE, $N = 6$) and mean cineole intake was 2.9 ± 0.3 g kg^{-1}. There was evidence of induction of secondary oxidative pathways, as hydroxycineoles were the major metabolites (48% total) on the first day, but rapidly dropped to 15% on subsequent days as the acid metabolites increased. These findings indicate that ingestion of cineole is not constrained by selective saturation of individual enzymes involved in its multiple pathways of oxidation, but rather the total detoxification capacity appears to limit feeding on a cineole diet.

Key Words—1,8-Cineole, metabolites, brushtail possum, *Trichosurus vulpecula*, detoxification, feeding.

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INTRODUCTION

Many studies concerned with the effect of plant secondary metabolites (PSMs) on diet selection in herbivores have speculated that generalist herbivores select a varied diet to ensure that a range of PSMs are ingested to avoid saturation of any one detoxification pathway (Freeland and Winter, 1975; Dearing and Cork, 1999). Although this hypothesis seems to be intuitively correct, we are only now beginning to understand the physiological processes involved in the biotransformation of PSMs such as monoterpenes. *In vitro* studies of microsomal preparations from the brushtail possum (*Trichosurus vulpecula*) have partially characterized the cytochrome P450 enzymes (CYP) involved in the oxidation of the monoterpenes 1,8-cineole (cineole) and *p*-cymene, and found both induction and competitive inhibition of enzymes (Pass et al., 1999, 2001, 2002; Pass and McLean, 2002). The *in vivo* metabolic fate of these terpenes, after single doses, has also been reported in detail by our group (Boyle et al., 1999, 2000a,b, 2001). In summary, the brushtail possum utilizes multiple enzymatic pathways to produce at least 19 oxidized metabolites of cineole with varying degrees of oxidation. There are 3 hydroxycineoles, 3 dihydroxycineoles, 2 cineolic acids, and 11 hydroxycineolic acids. The more extensively oxidized metabolites are mostly excreted unchanged, while the less oxidized metabolites are first conjugated with glucuronic acid before renal excretion. These single dose experiments have been critical for a preliminary understanding of the physiological fate of monoterpenes; however, the picture may be somewhat different in a chronic, voluntary feeding system.

Conclusions from feeding trials have been limited to correlations between dietary PSM concentrations and observational data such as food intake, urine and fecal output, and urinary pH and glucuronic acid (Foley, 1992; Guglielmo et al., 1996; Lawler et al., 1998, 2000; Dearing et al., 2000; Pass and Foley, 2000). We have now expanded this traditional approach by matching the metabolic outcome, described by the PSM detoxification products excreted in the urine and feces, with voluntary food intake.

We investigated chronic feeding by brushtail possums maintained on a diet containing the *Eucalyptus* leaf PSM, 1,8-cineole. Cineole is the most prevalent *Eucalyptus* terpene and is the major component (52.4%) in *Eucalyptus melliodora* (Boland et al., 1991), a preferred food for the brushtail possum. The oil content of *E. melliodora* varies from 2.9 to 4.2% dry weight (Morrow and Fox, 1980) or, allowing for 40% dry matter in the leaf (McLean et al., 1993; Boyle et al., 2001), 1.2–1.7% wet weight. The aims of the first experiment were (1) to establish whether cineole deters feeding when it is consumed in concentrations up to and exceeding those occurring in *Eucalyptus* diets, and (2) to identify saturation of any detoxification pathway associated with increasing cineole intake. Since cineole is metabolized via stepwise oxidations, we hypothesized that saturation of a specific detoxification enzyme would result in a decline in the proportion of the

metabolite product as cineole intake increased. Evidence for saturation of cineole detoxification pathways would provide support for the detoxification limitation hypothesis.

In the second experiment, naïve possums (defined as those that had not ingested any terpene for 2 months) were challenged with the maximum concentration of cineole used in the first experiment. The excretion of cineole metabolites was measured for evidence of induction of detoxifying enzymes during the early period of exposure. It was also expected that induction would result in an increase in the daily food and cineole intake.

METHODS AND MATERIALS

Animals. Approval for all animal experimentation was granted by the University of Tasmania's Ethics Committee (Animal Experimentation) and the Department of Primary Industries, Water and Environment (Tasmania).

Six brushtail possums (two females and four males) were trapped around Hobart, Tasmania. Possums were weighed regularly, and their weights remained relatively constant (3.6 ± 0.3 kg, mean $\pm$ SE) throughout their captivity.

Dosing and Urine Collection. Details of possum housing, metabolism cages, and procedures for collecting urine and fecal samples are given in Boyle et al. (2000a). Possums were fed an artificial diet prepared from freshly grated silverbeet (*Beta vulgaris*), carrot and apple, lucerne chaff, and sugar (McArthur et al., 2000), commencing several days before the experiments. Fresh food and water were supplied *ad libitum* and the daily intake of each was measured. Possums were transferred to metabolism cages ($60 \times 60 \times 45$ cm) at the start of each experiment.

The appropriate quantity of cineole was incorporated into the diet daily. To minimize loss by evaporation, cineole was first adsorbed onto the lucerne chaff before mixing the sugar and wet ingredients in order of bulk. Cineole concentrations are expressed as percentage wet weight of diet. The diet was divided into seven individual trays (six possums and one control) and weighed before being offered to the possums and again on removal the following day. The weight of food removed from the cages was adjusted appropriately to account for the weight lost via evaporation from the control tray of food. Each morning, remnant food was removed and weighed, the urine and feces were collected, and the next day's food was supplied. Therefore, urine analyses correspond to food and cineole intakes for the same 24-hr period.

Dosing Regimens. Experiment 1 was designed to establish the maximal daily intake and then demonstrate the antifeedant effect of cineole. Gradual incremental increases in dietary cineole concentrations were used, so all animals had the same exposure, ensuring maximum physiological adaptation to cineole. Possums spent 2 d adjusting to the metabolism cages and were fed the basal diet. Cineole (0.5%)

was added on the third day, and the concentration increased every second day in increments of 0.5% to a maximum of 4%.

In Experiment 2, Possums were maintained on a terpene-free diet for 2 mo between Experiments 1 and 2. They were readjusted to metabolism cages and fed the basal diet for 2 d before starting 4% cineole for 5 d. The basal diet was then reintroduced for a further 2 d in order to measure the time required to clear metabolites from the body after discontinuation of cineole. We refer to this period as metabolite "washout."

Metabolite Analyses. Representative urine samples from each experiment were analyzed as previously described (Boyle et al., 2000a). Briefly, diluted urine was acidified to pH 1 with 5 M HCl and then extracted with ethyl acetate. Extracts were doubly derivatized first to form methyl ester derivatives of carboxylic acid groups, and then trimethylsilyl ether derivatives of hydroxyl groups. Gas chromatography–mass spectrometry was used for identification and quantitation of cineole metabolites. Cineole metabolite standards used for quantitation had previously been isolated from urine.

Metabolites were measured before (free metabolite levels) and after (total metabolite levels) hydrolysis with *Helix pomatia* (β-glucuronidase 141,000 units/ml plus aryl sulphatase 3950 units/ml; Boehringer Mannheim, Germany). The difference between the two measurements indicates the extent of metabolite conjugation with glucuronic acid. Experiment 1 urine samples corresponding to the second day of the 0.5, 2.0, and 4.0% cineole treatments (days 4, 10, and 18, respectively) and Experiment 2 urine samples from days 3, 4, 5, 7 (4% cineole treatment), 8, and 9 (washout period) were selected for analysis. Feces were also collected on these days and analyzed for cineole and metabolites.

Interpretation of Metabolite Analysis. The biotransformation of cineole in brushtail possums is complex (Boyle et al., 2000a). Nineteen metabolites, encompassing varying degrees of oxidation, have been identified. Furthermore, many of these metabolites are excreted, at least partially, as their respective glucuronides. To simplify interpretation of metabolite analyses, metabolites were grouped based on the degree and type of oxidation. Where appropriate, the extent of conjugation was determined by comparing total (hydrolyzed urine) versus free (unhydrolyzed) metabolite recoveries.

Data Analysis. Data are presented as mean $\pm$ SE of six possums, unless otherwise indicated. The software Prism (GraphPad Software Inc, San Diego, CA) was used for graphing purposes as well as performing repeated measures and two-factor ANOVA and linear regressions on data.

RESULTS

Dietary Intake. Food intake decreased as cineole concentrations increased throughout Experiment 1 (Figure 1A; repeated measures ANOVA $F_{8,40} = 4.55$,

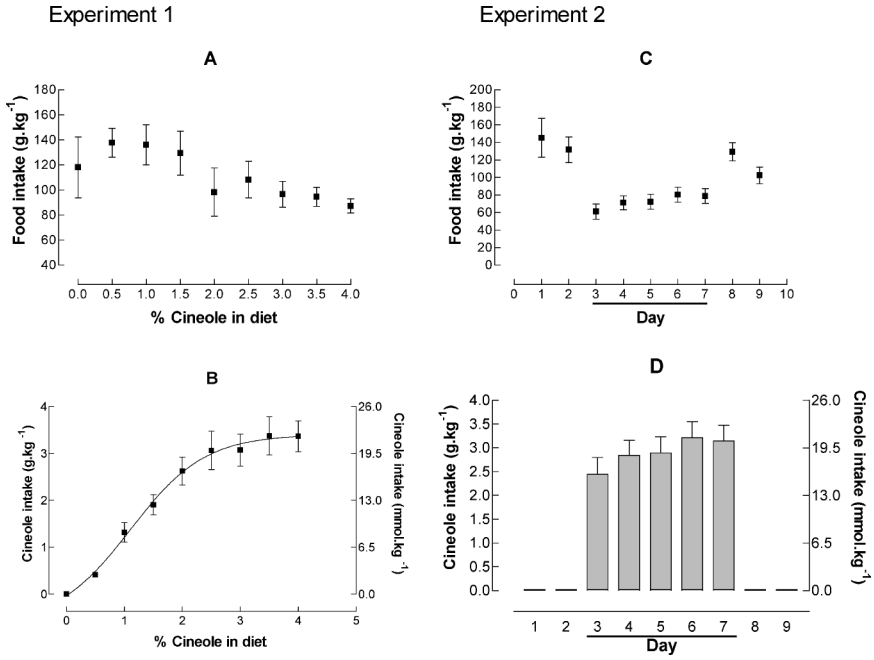

FIG. 1. (A) Food intake (g kg^{-1}) and (B) cineole intake (g kg^{-1} and mmol kg^{-1}; $N = 4$ possums) vs. dietary cineole concentration in Experiment 1. Dietary cineole concentrations were increased by 0.5% every second day, and the above values are calculated from the average intake of both days at each concentration. (C) Food intake (g kg^{-1}) and D) cineole intake (g kg^{-1} and mmol kg^{-1}) throughout cineole treatment days in Experiment 2. A diet of 4% cineole was introduced on day 3 and discontinued after day 7 in Experiment 2 (cineole days are underlined). Values reported as mean ± SE for $N = 6$ possums, except where stated.

$P < 0.0005$). Mean food intakes on control days of the experiment were highly variable. However, the variability in food intake was reduced after cineole (0.5%) was introduced. There was a 36 ± 4% decrease in food intake as animals progressed from the 0.5% diet (138 ± 12 g kg^{-1}) to the 4% diet (87 ± 6 g kg^{-1}).

Initially, cineole intake increased with increasing dietary cineole concentrations (Figure 1B). However, at dietary concentrations of 3% or more, daily cineole intake did not increase further than the maximum of 3.4 ± 0.2 g kg^{-1} ($N = 4$). The intakes of two of the six possums were not included in Figure 1B as their intake results were incomplete. One possum was withdrawn from the cineole treatment for 2 d (corresponding to 3% cineole) and instead offered 1.5% diet, due to concern

that this possum failed to defecate for three consecutive days as well as having a marked reduction in food consumption. After a 2-d "rest," the possum reestablished regular feeding and defecating, and was reinstated into the treatment regimen at 3.5% cineole and continued to the end of the experiment. A second possum regularly scattered the contents of its food bowl into the metabolism cage at cineole concentrations greater than 2%, making recovery of remnant food difficult and often incomplete. Interestingly, the maximum intake of cineole for individual possums ($N = 6$) occurring on any treatment day showed little variability (3.8 ± 0.2 g kg^{-1} or 5.2 ± 0.3 g $kg^{-0.75}$).

At low cineole concentrations, possums would consume some of the food immediately upon presentation. However, as the concentration increased, enthusiasm for investigating and eating food on presentation diminished. Generally, possums tended to scatter their food with increasing cineole, presumably looking for more palatable portions.

The abrupt introduction of the 4% cineole diet in Experiment 2 caused a $45 \pm 6\%$ decrease in food consumption. There was a gradual and significant increase in food and, therefore, cineole intake during the cineole treatment period (Figure 1C and D; repeated measures ANOVA $F_{8,40} = 7.84$, $P < 0.001$, *post hoc* test for linear trend $P < 0.05$). Possums consumed 28% more cineole on the final day of cineole treatment than on the first. The maximum daily intake of cineole recorded for each possum on any day was 3.3 ± 0.3 g kg^{-1} or 4.5 ± 0.4 g $kg^{-0.75}$. Cessation of the cineole resulted in an immediate recovery of food consumption. There was good consistency between the experiments in food and cineole intakes at 4% (Figure 1A–D).

Excretory Outputs. Urine volumes in Experiment 1 differed significantly between experimental days (repeated measures ANOVA $F_{8,40} = 5.39$, $P < 0.001$). However, there was no linear trend across the cineole concentration range (*post hoc* test for linear trend $P > 0.05$). Maximum urine output was 335 ± 95 ml (1.5% cineole diet) and the lowest output was 225 ± 42 ml (3.5% cineole diet). Urine pH decreased progressively from pH 8.2 ± 0.1 to pH 6.2 ± 0.4 as cineole intake increased. Fecal output also proved to be highly variable, and there was a nonsignificant trend towards reduced output with the higher concentrations of cineole (repeated measures ANOVA $F_{8,40} = 4.07$, $P = 0.07$; Figure 2A). At the higher concentrations of cineole, possums frequently failed to defecate, sometimes for a few days at a time.

In Experiment 2, there was a significant reduction in urine output with increasing food cineole concentration (repeated measures ANOVA $F_{8,40} = 7.06$, $P < 0.001$). There was a marked drop from pH 8.3 ± 0.1 to pH 6.1 ± 0.3 with cineole treatment. Fecal output was greatly reduced with the introduction of cineole to diet (Figure 2B).

Metabolite Analyses. The dietary concentration of cineole and corresponding intake for the days when urine samples were analyzed for cineole metabolites are

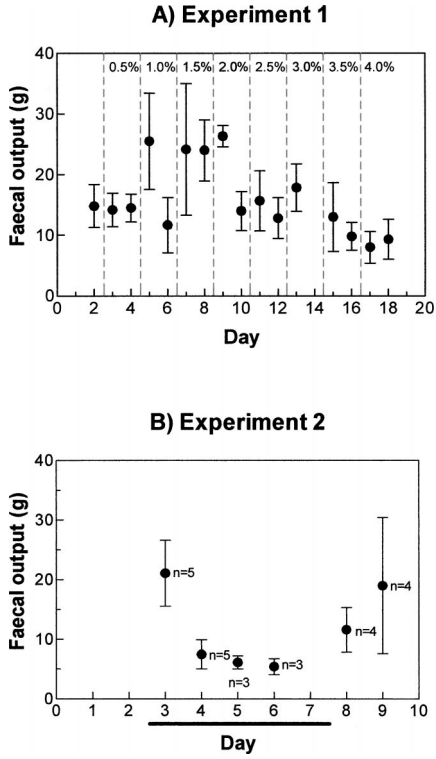

FIG. 2. (A) Fecal output (g) for each day of Experiment 1. Although not of statistical significance, there was a trend suggesting a reduction in feces production as the cineole concentration increased (repeated measures ANOVA $F_{8,40} = 2.00$, $P = 0.07$). (B) Fecal output (g) for days 3–9 of Experiment 2 (cineole treatment days are underlined). Fewer possums produced fecal pellets, and fecal weights were reduced during the treatment period. Values reported as mean $\pm$ SE for $N = 6$ possums.

summarized for each experiment in Table 1. All 19 metabolites previously reported for cineole in the brushtail possum (Boyle et al., 2000a) were present in possum urine in the two experiments.

Experiment 1. The fraction of ingested cineole recovered as metabolites did not vary among the three sample days for free or total metabolites (Table 2). The excretion pattern of metabolite groups, after hydrolysis, throughout the experiment is shown in Figure 3, as the percentage of urinary metabolites recovered. The relative proportion of each metabolite group did not change significantly as cineole concentration increased (repeated measures ANOVA for hydroxycineoles $F_{2,10} = 0.12$, $P = 0.89$; cineolic acids $F_{2,10} = 2.30$, $P = 0.15$; dihydroxycineoles $F_{2,10} = 0.62$, $P = 0.56$; or hydroxycineolic acids $F_{2,10} = 0.55$, $P = 0.60$).

TABLE 1. DAYS ON WHICH URINE ANALYSES WERE PERFORMED FOR EACH EXPERIMENT AND THE CORRESPONDING DIETARY CINEOLE CONCENTRATIONS (% WET WEIGHT) AND CINEOLE INTAKES

Experiment 1 cineole intake				
Unit	Day 4 (0.5% cineole)	Day 10 (2.0% cineole)	Day 18 (4.0% cineole)	
g	2.4 ± 0.2	5.5 ± 0.9	12.2 ± 1.2	
mmol	15.8 ± 1.5	35.4 ± 5.7	79.0 ± 7.9	
mmol/kg	4.5 ± 0.4	10.3 ± 1.8	22.3 ± 1.7	
Experiment 2 cineole intake				
	Day 3 (4.0% cineole)	Day 4 (4.0% cineole)	Day 5 (4.0% cineole)	Day 7 (4.0% cineole)
g	8.8 ± 1.5	10.14 ± 1.4	10.1 ± 1.2	10.9 ± 0.9
mmol	57.0 ± 9.9	65.8 ± 9.4	65.8 ± 8.0	71.0 ± 6.0
mmol/kg	15.9 ± 2.3	18.4 ± 2.1	18.8 ± 2.2	20.4 ± 2.1

Notes. Values reported as mean ± SE, $N = 6$. Days 1, 2, 8, 9 = zero intake of cineole. Day 6 = 4% cineole (no measurements taken).

Overall, the hydroxycineolic acid metabolites accounted for 56% of the total (hydrolyzed) excreted metabolites. Cineolic acids were the next most abundant group (27%), then hydroxycineoles (13%), and dihydroxycineoles (4%) accounted for the remaining metabolites.

Figure 4 shows the molar recovery of each metabolite group before and after hydrolysis. The difference between the total and free levels represents hydrolyzable conjugation. Hydrolysis increased the recovery of hydroxycineoles, cineolic acids, and, consequently, total metabolite recovery (two-way ANOVA $F_{1,30} = 11.75$, $P = 0.002$; $F_{1,30} = 12.11$, $P = 0.002$; and $F_{1,30} = 4.80$, $P = 0.04$, respectively), but did not affect the dihydroxycineole or hydroxycineolic acid groups ($F_{1,30} = 2.54$, $P = 0.10$; $F_{1,30} = 0.87$, $P = 0.36$, respectively).

TABLE 2. FRACTIONAL RECOVERY OF CINEOLE INTAKES AS URINARY METABOLITES IN EXPERIMENT 1

	Day 4 (0.5%)		Day 10 (2.0%)		Day 18 (4.0%)	
	Free	Total	Free	Total	Free	Total
Mean ± SE ($N = 6$)	0.31 ± 0.05	0.44 ± 0.06	0.37 ± 0.06	0.55 ± 0.09	0.23 ± 0.05	0.40 ± 0.06

Note. Metabolite recoveries increased after hydrolysis for each day (two-factor ANOVA $F_{1,30} = 10.3$, $P = 0.003$). The fraction of the ingested cineole recovered as metabolites did not vary between the three sample days for either free or total metabolites (repeated measures ANOVA $F_{2,10} = 2.03$, $P = 0.18$; and $F_{2,10} = 1.13$, $P = 0.36$, respectively). The difference between total and free represents the fraction excreted as conjugates.

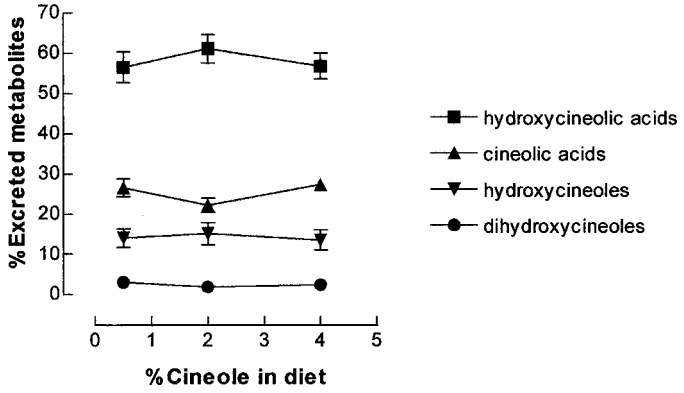

FIG. 3. Recovery of cineole metabolites as a percentage of total urinary metabolites in Experiment 1 (mean ± SE, $N = 6$).

The percentage of total metabolites excreted as glucuronides on the 0.5, 2.0, and 4.0% diets was 28 ± 6%, 30 ± 5%, and 41 ± 9%, respectively, and these values did not vary significantly among the three sampling days (repeated measures ANOVA $F_{2,10} = 0.83$, $P = 0.47$).

Urinary glucuronic acid measurements were 446 ± 150 μmol/kg on the 0.5% cineole diet, 1735 ± 539 μmol/kg on the 2% diet, and 3087 ± 522 μmol/kg on the 4% diet. There was a reasonable correlation between dietary cineole concentration and urinary glucuronic acid excretion ($R^2 = 0.78$, $F_{1,16} = 56.4$, $P < 0.001$). There was also reasonable correlation between the molar amounts of conjugated metabolites excreted and the molar amounts of urinary glucuronic acid ($R^2 = 0.58$, $F_{1,16} = 22.0$, $P < 0.001$).

Experiment 2. Urine samples from days 3, 4, 5, 7, 8, and 9 were analyzed for urinary metabolites and glucuronic acid. Cineole intakes for these days as well as the treatment regime of cineole are summarized in Table 1.

The fraction of ingested cineole recovered as both free and total metabolites is reported in Table 3. The fractional daily recovery of total metabolites started very low and increased throughout the treatment period. As expected, the fractional recovery of total metabolites was significantly greater than the recovery of free metabolites on each day of the treatment.

As in Experiment 1, the overall pattern of excretion of metabolite groups was examined as the percentage of total recovered metabolites. Figure 5 shows a transition in the pattern of metabolite excretion during the first 3 d of the cineole diet. On the first day, the hydroxycineoles accounted for about 48% of metabolites but, over the following 2 d, this dropped to about 15%, and the percentage of cineolic acids and hydroxycineolic acids increased correspondingly. Once stabilized, the percentages of metabolites in each group were comparable to those reported in

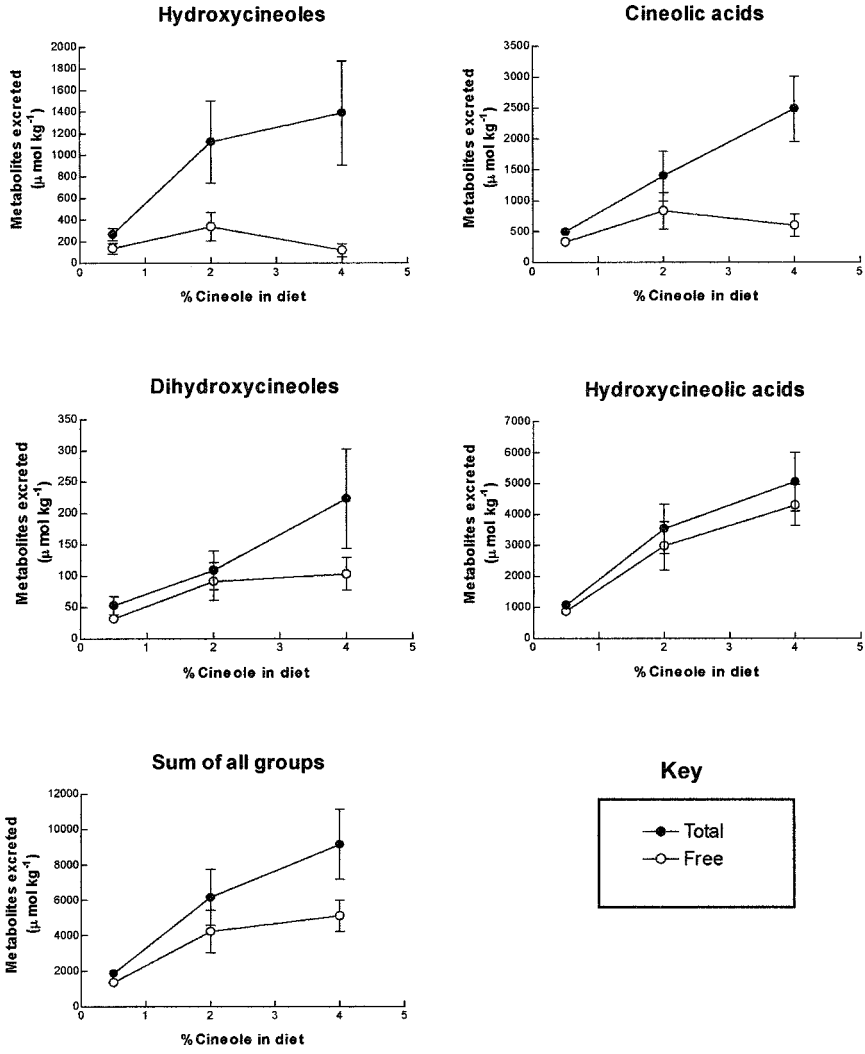

FIG. 4. Comparison of free and total metabolites for each metabolite group in Experiment 1. Values are μmol kg^{-1} (mean $\pm$ SE, $N = 6$).

Experiment 1. A similar pattern of metabolite excretion continued into day 8, the first washout day. During the second washout day (day 9), the percentage excreted as hydroxycineolic acid dropped while the percentage as cineolic acid rose.

Figure 6 shows the molar recoveries of free and total metabolites for each group. Only total metabolites were measured during the washout days (days 8

TABLE 3. FRACTIONAL RECOVERY OF CINEOLE INTAKES AS URINARY METABOLITES IN EXPERIMENT 2

	Day 3		Day 4		Day 5		Day 7	
	Free	Total	Free	Total	Free	Total	Free	Total
Mean ± SE (N= 6)	0.05 ± 0.02	0.09 ± 0.02	0.12 ± 0.02	0.20 ± 0.05	0.09 ± 0.02	0.24 ± 0.08	0.24 ± 0.04	0.46 ± 0.04

Note. Hydrolysis caused an increase in metabolite recovery each day (two-factor ANOVA $F_{1,40} = 17.52$, $P < 0.001$). The fraction of the ingested cineole recovered as total metabolites increased significantly throughout the treatment period (repeated measures ANOVA $F_{2,10} = 20.25$, $P < 0.001$).

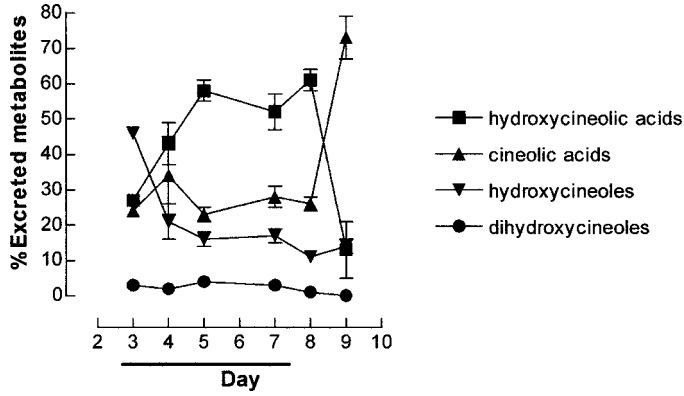

FIG. 5. Recovery of cineole metabolites as a percentage of total urinary metabolites in Experiment 2 (mean ± SE, $N = 6$). Cineole treatment days are underlined.

and 9). In the first 24 hr after cineole cessation, significant amounts of each metabolite group were measured in the urine. Forty-eight hours after cineole was discontinued, metabolite excretion had diminished to insignificant levels.

Hydrolysis resulted in significant increases in the recovery of hydroxycineoles, cineolic acids, dihydroxycineoles, and total metabolites (two-way ANOVA $F_{3,40} = 20.88$, $P < 0.001$; $F_{3,40} = 16.78$, $P < 0.001$; $F_{3,40} = 4.20$, $P = 0.05$; and $F_{3,40} = 4.08$, $P = 0.002$, respectively). Recovery of hydroxycineolic acids did not increase after hydrolysis ($F_{3,40} = 1.26$, $P = 0.27$).

The percentage of metabolites excreted as glucuronides on days 3, 4, 5, and 7 were 48 ± 9%, 34 ± 10%, 60 ± 9%, and 49 ± 5%, respectively ($N = 6$, repeated measures ANOVA comparing fraction of conjugated metabolites between the four sampling days $F_{3,15} = 1.80$, $P = 0.19$). The pattern of conjugation for individual metabolites belonging to the hydroxycineole, cineolic acid, and dihydroxycineole groups reflected the overall conjugation of their respective groups in each experiment. The least oxidized metabolites, the hydroxycineoles, underwent the greatest degree of conjugation.

Urinary glucuronic acid increased significantly throughout the cineole treatment period from 389 ± 140 μmol/kg on the first treatment day to 3608 ± 963 μmol/kg on the final day of cineole treatment (repeated measures ANOVA $F_{3,15} = 8.09$, $P = 0.002$).

Analysis of fecal pellets collected in Experiment 2 found no unchanged cineole. However, cineole metabolites were present, and the daily total fecal metabolite output is shown in Figure 7. Fecal excretion of metabolites was delayed and also prolonged beyond cessation of the cineole treatment. Because of this delay, it was not possible to determine the overall fraction of ingested cineole excreted as fecal metabolites. However, from the metabolites recovered, only a small fraction (<1%)

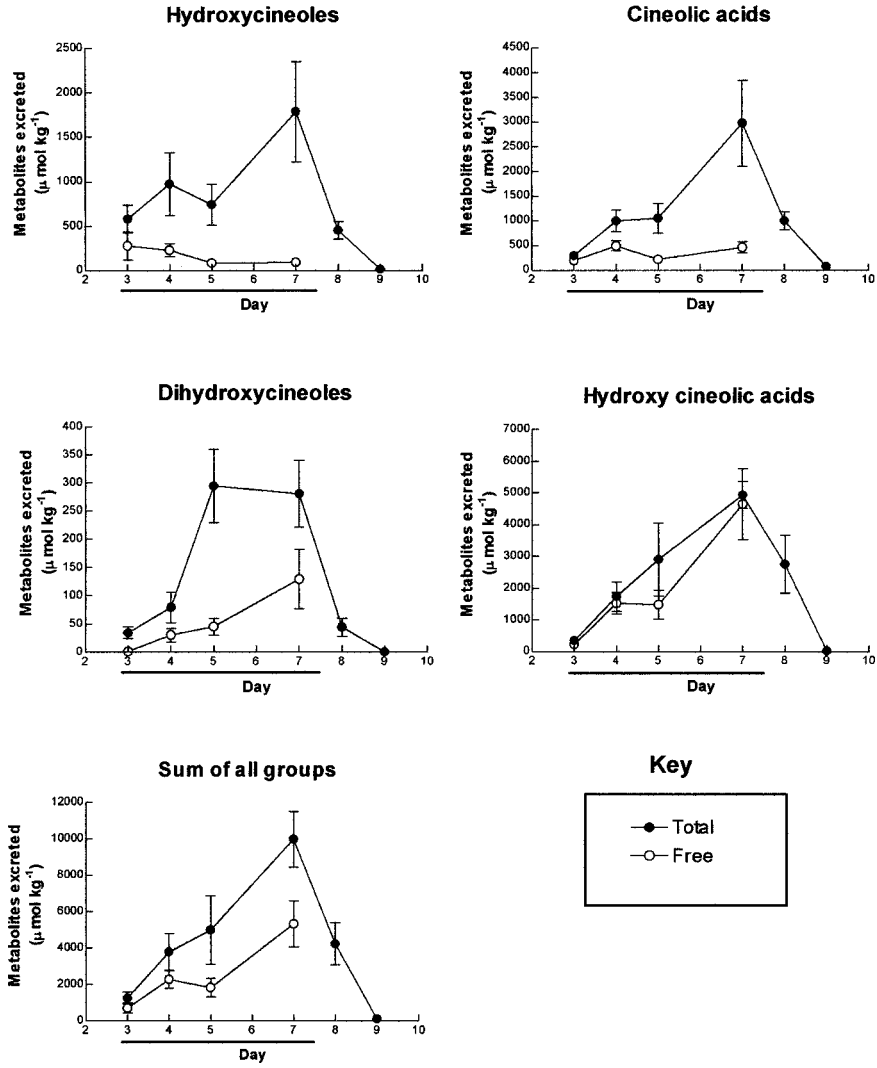

FIG. 6. Comparison of free and total metabolites for each metabolite group in Experiment 2. Values are μmol kg^{-1} (mean $\pm$ SE, $N = 6$). Cineole treatment days are underlined. Only total metabolites were measured during the washout period (days 8 and 9).

of cineole was excreted by this route up to the end of day 9. Hydroxycineolic acid metabolites accounted for 89.2 $\pm$ 3.6% of recovered fecal metabolites, dihydroxycineoles accounted for 5.9 $\pm$ 3.3%, cineolic acids 3.5 $\pm$ 1.5%, and hydroxycineoles 1.5 $\pm$ 0.3%. Hydrolysis of fecal samples did not increase the recovery of any

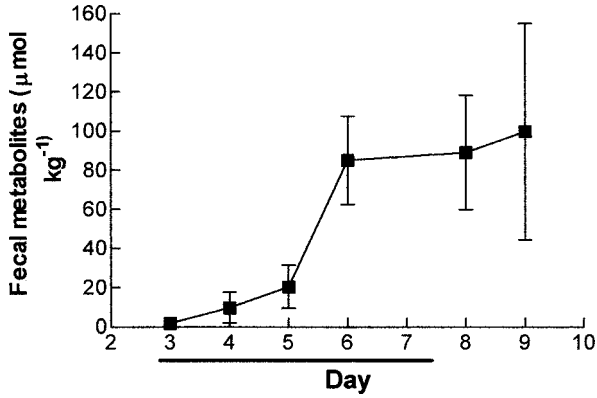

FIG. 7. Total molar recovery of fecal metabolites (μmol kg^{-1}) for each day of analysis in Experiment 2. Values are reported as mean $\pm$ SE and the cineole treatment days are underlined.

group of metabolites (Student's paired *t*-test $P > 0.05$ for each group). Comparison of the metabolite patterns in fecal and urine extracts indicates that the less oxidized metabolites were under represented in feces.

DISCUSSION

This is the first report of a PSM feeding experiment to directly examine detoxification products in an attempt to determine the processes that underly feeding constraints. By quantifying detoxification products, we were able to show, *in vivo*, that induction of oxidation enzymes occurs after initial exposure to cineole. Another important finding was that there was no evidence from the detoxification products to suggest saturation of the enzymes responsible for individual oxidation pathways at the maximum daily intake of cineole.

We have demonstrated that possums will not voluntarily ingest cineole above a critical maximum daily amount. Four of six possums progressively increased their cineole intake to a maximum that was associated with decreased food consumption and, while the remaining two possums had a more erratic feeding pattern, their maximum cineole intake was approximately the same. The small variation between animals in the maximum cineole ingested suggests that possums were truly being constrained by the high cineole concentration rather than other factors.

In a similar feeding experiment, Lawler et al. (1999) reported no limit to the amount of cineole possums were able to ingest, up to an intake of 3.5 g kg$^{-0.75}$ of cineole. We increased the concentrations of cineole offered to our possums well

beyond this level and finally observed the maximum intake of cineole at 5.2 ± 0.3 g $kg^{-0.75}$.

Induction. The rise in the proportion of more highly oxidized hydroxycineolic acid metabolites during the first days of cineole exposure in Experiment 2 is evidence of induction, particularly those enzymes responsible for secondary oxidations (Parkinson, 2001). The same transition in metabolite excretion pattern was not seen in Experiment 1, most likely due to the initial analysis being performed on the second day of the low cineole diet.

Also in Experiment 2, food consumption increased by 28%, and the majority of the increase occurred over the first day. Although behavioral adaptation may account for some of the increase, we would expect intake to increase as enzyme capacity increased. Naïve possums appear to have a baseline capacity for oxidizing cineole. However, induction of the detoxification enzymes would increase detoxification capacity and result in an associated increase in food intake.

Induction of terpene oxidation enzymes in eucalyptus folivores was initially suggested after the observation that common ringtail possums (*Psuedocheirus peregrinus*) gradually increased their intake of a newly introduced eucalyptus leaf over several days (McLean et al., 1993). Terpene-induced induction of oxidation of cineole by cytochrome P450 enzymes has also been demonstrated in brushtail possum liver microsomal preparations (Pass et al., 1999).

Saturation. We hypothesized that, if feeding is limited by saturation of any particular detoxification enzyme, then the proportion of metabolite produced by that enzyme would decline as cineole intake increased beyond the level of saturation. However, the relative amounts of the four groups of metabolites remained constant over an eightfold range of dietary cineole concentrations, including concentrations that reduced food consumption (Experiment 1). In Experiment 2, the relative proportions of each metabolite group were also similar to those in Experiment 1 once induction was established. There was no evidence of selective saturation of the enzymes responsible for particular oxidative pathways, even at the highest intakes of cineole. Without administering higher doses, we cannot determine whether the maximum cineole intake reflects the maximum enzyme capacity beyond which saturation occurs.

Interestingly, the capacity to metabolize cineole greatly exceeds the likely daily maximum intake of this PSM from a foliar diet, as previously concluded by Lawler et al. (1999). There are, however, a number of differences between an artificially contrived diet and a natural foliar diet. First, the nutritional quality of the artificial diet, which is based on fruit and vegetables, is likely to be better than that of a leaf diet. The energy and substrates (e.g., glucose) required to power detoxification reactions are also likely to be available in excess. Furthermore, in these experiments, all resources can be diverted into the detoxification of the single PSM. In a natural leaf-feeding situation, a multitude of PSMs often require detoxification at the same time. Complexities such as induction as well as

competitive inhibition of oxidation enzymes may complicate the ability to detoxify and eliminate PSMs. For example, the *in vitro* metabolism of cineole is competitively inhibited by the other *E. melliodora* terpenes, limonene, α-pinene, and *p*-cymene (Pass and McLean, 2002).

Glucuronidation. The basal diet alone produced only minor amounts of urinary glucuronic acid. As cineole intake increased in Experiment 1, there was a proportional increase in urinary glucuronic acid excretion, up to 3 g per day. Glucuronidation was more important in the elimination of the less oxidized metabolites, presumably because the unconjugated metabolites were too lipophilic for efficient renal clearance (Parkinson, 2001).

Urine Acidity. Urine pH decreased by as much as 2 units as a direct result of the excretion of acidic cineole metabolites. The formation of acidic metabolites results in an acid load that, by challenging acid–base homeostasis, could limit cineole consumption (Foley et al., 1995).

Metabolite Recovery. Recovery of the estimated cineole intake for all experiments was consistently low throughout each experiment (Tables 2 and 3). A likely cause of these low recoveries is an overestimation of intake. Cineole intakes were calculated from food intake and the cineole concentration. Although cineole is not highly volatile (bp 176–177˚C), a variable amount evaporates depending on degree and frequency of food disturbance and the surface area of exposed food. Furthermore, it was difficult to retrieve all remnant food for inclusion in the residual weight due to animal scattering of food. The effect of overestimating cineole intake is limited to the fractional recovery as metabolites. The pattern of metabolite excretion is unaffected by the actual intake of cineole.

We have reported in a previous study that cineole is excreted by the possum in expired air, although the amount was not quantitated (Boyle et al., 2002). However, human studies have found that about 8% of inhaled α-pinene is expired unchanged (Falk et al., 1990; Levin et al., 1992).

Fecal Metabolites. Experiment 2 showed that fecal excretion of metabolites was delayed by 2–3 d and continued well after cineole exposure was discontinued. The highest concentration of metabolites occurred 2 d after cineole ceased, and more sampling days would be required to determine the length of time that metabolites were excreted in the feces. The lag in fecal excretion is not surprising given the reduction in fecal output observed at the onset of cineole treatment.

Usually, metabolites would need to be conjugated to be excreted in bile, as only larger molecular weight compounds (>325) are secreted by this route (Rozman and Klaasen, 2001). However, fecal metabolites were excreted in their free form, indicating that hydrolysis of metabolites occurred *in vivo*, probably by gut flora.

Metabolite Washout. Despite the size of the ingested cineole dose, possums were capable of eliminating it rapidly. The absorption, metabolism, and excretion of dietary cineole and its metabolites was essentially complete within 48 hr of discontinuing the diet. The change in pattern of metabolites on the second washout

day (Figure 5) is not particularly significant, as the amounts excreted that day were very small (Figure 6).

Food and Cineole Intake. The estimated amounts of cineole ingested in these experiments were substantial—up to 12 g per day. This represents a major detoxification challenge, given the relatively small size of brushtail possums (mean weight 3.6 kg). From the insights into detoxification processes that we have acquired in this study, it is clear that possums have a very high capacity to cope with large amounts of dietary cineole, at least when it is the sole PSM present.

Urinary and Fecal Output. Introduction of the 4% cineole diet reduced urine and fecal output for all possums. This probably reflects reduced food intake. It is assumed that possums will not voluntarily ingest cineole beyond their detoxification capacity, and that this limits the voluntary intake of cineole. The limiting factor may be the total capacity for cineole metabolism, for example the supply of energy to the CYP enzymes. However, this would also limit the capacity to metabolize other terpenes, and there would be no advantage for the animal to switch to another leaf containing different terpenes. The antifeedant stimulus may be the accumulation of cineole, or its metabolites, or both. Other factors may contribute to the antifeedant effect of cineole, as shown in the change in observed feeding behavior as dietary cineole increases. Increasing cineole concentration apparently reduces palatability as evidenced by possums showing less interest in food as well as tending to scatter it.

How these findings affect the widely regarded hypothesis, that generalist herbivores select their leaf diet to ensure that small quantities of a wide range of PSMs are ingested to avoid saturation of enzymatic detoxification pathways (Freeland and Janzen, 1974), is unclear. We have shown that the maximum daily voluntary cineole intake does not result in saturation of selective detoxification pathways, and yet under optimal conditions, the capacity for detoxifying cineole far exceeds the typical daily intake from a foliar diet.

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MALE URINARY CHEMOSIGNALS DIFFERENTIALLY AFFECT AGGRESSIVE BEHAVIOR IN MALE MICE[1]

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Abstract—Chemical signals modulate aggressive behavior in mice. For example, male urinary cues enhance aggression against other adults: a resident mouse attacks a male but not a castrated intruder, unless it is anointed with male urine. Our purpose was to understand whether molecules excreted with urine also act as aggression triggers in a different context. Therefore, the effect of urine, or molecules purified from urine, voided by different animals (males or females), was tested on the aggression of male mice against pups. Latency to the first attack, percentage of pups receiving the first attack, and percentage of attacked pups after 5 and 15 min were recorded. At variance with intermale aggression, male urinary chemosignals sprayed on pups reduced infanticide, while female urine did not. Male urine also delayed infanticide when compared to female urine. Pups anointed with low molecular weight dialyzed urine and with the high molecular weight protein fraction were attacked later than controls. Pups anointed with Major Urinary Proteins (MUPs) also were attacked later. The volatiles retained by MUPs act in the same way as adult male urine. MUPs and their ligands did not modify biting of food items. The results show that mice do not perceive male chemosignals as compulsory aggression triggers but rather can consistently and differentially shape their behavior in response to the same molecules according to different contextual events.

Key Words—Mice, pheromones, infanticide, lipocalins, urine, aggression, Major Urinary Proteins, behavior, androgens.

[1]The editors are aware of the ethical questions raised by this work. The authors, however, complied with all applicable rules and regulations of their home country and obtained all necessary legal authorizations to do the work.

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INTRODUCTION

Mice have a well-developed olfactory system, and rely largely on chemical signals for shaping their behavior in response to changing external stimuli. A pivotal role in intraspecific communication is played by urinary chemosignals that provide information on the emitters (Keverne, 1983; Halpern, 1987; Brennan and Keverne, 1997). Male urine is attractive for females and repellent for other males (Jones and Nowell, 1974; Mossman and Drickamer, 1996). Several androgen-dependent molecules act as male cues, and a peculiar role is played by the Major Urinary Proteins (MUPs) and their ligands. Both components of the MUP/ligand complex appear to activate the vomeronasal organ (Brennan et al., 1999; Leinders-Zufall et al., 2000), and to induce different effects on reproductive physiology (Vandenbergh et al., 1976; Mucignat-Caretta et al., 1995; Brennan et al., 1999; Marchlewska-Koj et al., 2000), on exploratory behavior (Mucignat-Caretta et al., 1998, 1999a; Mucignat-Caretta, 2002), and on territory marking (Hurst, 1990; Hurst et al., 1998). MUPs participate also in individual recognition (Hurst et al., 2001). MUP-borne volatile molecules modulate agonistic interactions.

Olfactory pathways project to different amygdala nuclei involved in aggression (Novikov, 1993). Lesions of the medial amygdaloid nucleus, which receives inputs from the accessory but not the main olfactory bulb, affect social aggression (Luiten et al., 1985). Lesions of both olfactory bulbs result in an enhancement of predatory killing and infanticide, but in a decrease of social aggression (Albert and Walsh, 1984), while a reduction of infanticide has been observed after removal of the vomeronasal organ (Mennella and Moltz, 1988b). Therefore, olfactory systems are relevant in mediating both aggression toward adults and conspecific pups.

Under some conditions, olfactory modality seems to overcome other sensory input; a resident male mouse in his cage attacks a strange male intruder, while a nonaggressive behavior is shown toward a castrated male or a female. The latter behavior is reversed when the castrated male or the female is sprayed with male urine (Mugford and Nowell, 1970), suggesting that the most important signal for a male mouse is represented by chemicals present in male urine. Other investigations have provided a better chemical characterization of these aggression-promoting substances (Novotny et al., 1985; Mucignat-Caretta and Caretta, 1999b). They are androgen-dependent volatile molecules carried in the urine of adult males and slowly released in the environment by the MUP complex (see Bacchini et al., 1992; Cavaggioni and Mucignat-Caretta, 2000).

As with much behavior in mice, infanticide is influenced by olfaction. Prior contact with the mother reduces killing of her pups (Huck et al., 1982), as does prolonged contact with a pregnant female behind a mesh (Palanza and Parmigiani, 1991). Since different mechanisms modulate aggressive behavior (Ferrari et al., 1996), we speculated that chemical signals also could differently affect a variety of aggression paradigms. The aim of the present work was to investigate the effect

of male urine or urinary molecules on aggressive behavior of male mice toward pups, and to clarify the role of male chemosignals in regulating different kinds of aggression. Sudden attacks against newborns of the same species, often resulting in harm and infanticide, appear in adult male mice, and are enhanced by isolation (Huck et al., 1982). Intermale aggression and infanticide are related and covarying, and Swiss mice show a high level of both (Parmigiani et al., 1999). Infanticide is thought to be an adaptive mechanism, since destruction of litters accelerates the return to oestrus cycles of the mother. A specific inhibitory mechanism has been reported for litter survival (Perrigo et al., 1991) that is influenced by both mating and postmating social experience (Elwood, 1985; Kennedy and Elwood, 1988).

The present experiments were designed to test whether male urinary cues also enhance male aggression toward newborns, as toward adult mice. If male chemosignals are aggression triggers, they should also act by enhancing aggression toward pups, either by reducing the latency to attack or by directing more attacks toward male-smelling pups.

METHODS AND MATERIALS

Subjects. Adult Swiss male mice were housed in standard plastic cages (42 $\times$ 26 $\times$ 15 cm), with food (mouse food pellets, Mucedola, Italy) and water freely available, under a 12-hr L:D cycle (lights on at 0600 hours). Temperature was $24 \pm 1^{\circ}$C. Behavioral testing took place 1 hr before the onset of the dark period. All animals were weaned at 21 d of age, then lived in groups ($N = 6$) of the same sex; mice from two different litters (born within the same 24 hr) were mixed, three mice from each litter. One month before testing, males were isolated in cages as above and tested around 4 mo of age. Males that showed no attacks during the test were considered noninfanticidal and discarded from the experiment. Usually, less than 10% of isolated mice were noninfanticidal. To avoid repeated exposure to similar substances and to avoid learning effects, different males were tested in each experiment. To reduce variability, pups from the same litter were used for each tested mouse. A large variation in latency to the first attack was observed when retesting the same animal (see Results section), therefore, in order to test multiple substances on the same animal, it was necessary to test them simultaneously, to avoid misinterpretation due to repetition of testing on the same animal. Experiments were carried out in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC, Italian law 116/92). Experimental procedures were approved by the Italian Ministry of Health.

Behavioral Assays: Experimental Setup. Male mice were tested in their home cages. The cage lid was lifted, and two (or three in some experiments) pups were introduced in opposite corners. Since the spatial arrangement of stimuli could interfere with results, the two chosens corners were opposite the nest site of the

resident mouse. This was easily detected since the wood shavings litter was not changed in the 72 hr preceding the test. When three pups were tested, the three corners that did not host the nest site were used. The position of control pups was changed with respect to nest location from mouse to mouse. Within each experiment, no effect of location from nest site was apparent, the choice of pup being consistent with its smell and not with its position in the cage.

Behavioral Assays: Pup Scenting. Pups were newborns, with a maximum age of 48 hr. Unless otherwise stated, one pup was an untreated control, the other was painted with a test substance. Before entering the male's cage, the control pup rested on the control spoon, while the test pup was put for 30 sec on a glass dish containing 200 μl of the test substance. Test substances were adult male urine, adult female urine, adult pregnant female urine, dialyzed male urine, concentrated male urine, or MUPs. Both pups were manipulated with different spoons in order to avoid cross-contamination of odors.

Behavioral Assays. After the introduction of pups, mice were videotaped for a maximum of 15 min, or until both pups received one attack. The presence of the human observer was reduced as much as possible, with the experimenter always out of sight of the mouse, covered by a labcoat and wearing gloves and a mask, downwind with respect to the controlled air flow of the experimental room, and behind the light source. An experimenter, unaware of the pup treatement, collected the following data: the latency to first attack (a bite or sequence of bites) on each pup; number of mice attacking in the first 5 min and at the end of the test, and type of pup that received the first attack. If a mouse failed to show aggressive behavior, it was assigned the maximum latency (900 sec).

Test Design and Animal Care. The test was designed according to Elwood (1991) and Elwood et al. (1991). Protective screens that prevent contact with the pups were not used since the aim was to test the role of volatile and nonvolatile molecules as well. Care was taken to minimize the sufferings of harmed pups, by administering, as soon as the first attack ended, an overdose of anesthetic (xilazine 40 mg/kg body weight and ketamine 150 mg/kg body weight). The harmed pup was removed when the test mouse finished its first attack: this meant that the mouse moved away from the pup site and began other activities (grooming, sleeping, or digging), so that it was left undisturbed and apparently unaware of the rapid disappearance of the pup. Unharmed pups were promptly returned to their mother and not retested. None of them, either scented or unscented, was rejected by the mothers.

Experimental Design. Table 1 presents a summary of the conditions used in the different experiments. In experiment 1, 17 males were tested by simultaneously presenting a pup anointed with adult male urine and a control unscented pup. In experiment 2, a control and a pup painted with pooled female urine were presented to 15 males to test whether the effects seen in experiment 1 were specific for male cues. Since a protective effect of pregnant female urine has been observed in rats

TABLE 1. EXPERIMENTAL CONDITIONS OF THE EXPERIMENTS

Experiment	Adult mice tested	Pups presented	Conditions
1	17	2	Control vs. male urine
2	15	2	Control vs. female urine
3	15	2	Female urine vs. male urine
4	8	3	Control vs. dialyzed vs. concentrated
5	15	2	Control vs. MUPs
6	14	3	Control vs. MUPs vs. male urine
7	12	2 breadsticks	Control vs. MUPs

(Mennella and Molz, 1988a), a preliminary trial on eight mice was conducted on pups painted with pregnant mice urine, along with a control. In experiment 3, 15 mice were tested with two pups anointed either with male or with female urine to directly compare both stimuli. In experiment 4, to understand which urinary chemical stimuli were active, eight males were tested with three pups presented at a time, one unpainted control, one painted with male urine dialyzed with a 3000-Da filter and hence devoid of proteins, and a third painted with the urine concentrated above the filter and hence rich in proteins (the liquid phase being the same that crossed the filter). The experimental design was changed to include three pups in order to compare directly the strength of the effect of the different substances. In this way, we could record the order in which pups were attacked, in addition to the latency to attacks. Since concentrated urine also was active, we asked whether this was due to the subset of urinary odorants bound and released by MUPs. In other tests, MUP-borne volatiles are male cues that promote aggression against adults (Mucignat-Caretta and Caretta, 1999b). In experiment 5, 15 males were tested with a control pup and a pup anointed with purified MUPs; this experiment is similar to a preliminary experiment previously reported (Cavaggioni et al., 2001) that gave similar results. In experiment 6, we asked whether MUP-borne volatiles are active as whole intact urine. Fourteen males were tested with three pups, one control, one painted with MUPs, and one painted with adult male urine. In experiment 7, to test for a nonspecific aversive effect of MUPs, two food items were presented to 12 male mice, instead of pups. One piece of breadstick (3×1 cm, roughly similar to the size of a pup) was sprayed with MUPs, and the other was left untreated. In the other experiments, mice were not food deprived before testing. The latency to the first sniff and the latency to the first bite were recorded. In all other respects, this experiment was similar to the others. Mice had not previously encountered breadsticks nor newborn mice before.

Data Analysis. Data on the number of pups that were attacked in each group were analyzed with a χ^2 test: the number of control mice that were attacked after 300 and 900 sec was compared with the number of scented mice, attacked

after 300 and 900 sec. Absolute values on latencies were analyzed using the Statistica software. Since in some of the experiments data deviated from normality due to nonattacking mice that were assigned the maximum latency, data were analyzed with a Wilcoxon's test or, when three pups were used, with Friedman's nonparametric ANOVA, followed by Wilcoxon's test to explore the differences between two groups.

Urine Collection and MUPs Purification. Urine was collected from adult male or female mice. Sixty adult mice, aged 3–5 mo, served as urine donors. Urine from pregnant females was collected 16–18 d after mating. Untreated urine was collected by putting a mouse singly in a cage with a wire mesh grid over the floor for a maximum of 30 min and collecting the urine drops from the floor as soon as they were released, storing them at -20°C and thawing upon use. For biochemical preparations, male urine was collected overnight from metabolic cages in glass vials, and frozen until use. The dialyzed and concentrated fractions were prepared at the same time by thawing and filtering urine, then by concentrating with N_2 pressure over a membrane (molecular weight cutoff 3000 Da), the dialyzate being the fraction flowing through the membrane. MUPs were purified according to Cavaggioni et al. (1990). Upon thawing, male urine was centrifuged and paper filtered, concentrated by pressure over a membrane (molecular weight cutoff 10,000 Da), and chromatographed through a G50 (Pharmacia, Milan, Italy) molecular sieve column (1.5 cm inner diam, 60 cm long, 4-ml/hr flow). The fractions containing MUPs were pooled and again concentrated to reach a physiological concentration (10 mg/ml). Protein concentration was measured with a BioRad colorimetric protein assay. Homogeneity of protein was tested with poliacrylamide gel electrophoresis, showing one band of apparent molecular weight 19 kDa. The presence of MUP ligands was tested by gas chromatography, and showed the typical profile of volatile ligands (Mucignat-Caretta et al., 1995; Marchlewska-Koj et al., 2000).

RESULTS

Why Presenting the Male with Multiple Choice in Each Trial? A preliminary test on seven isolated adult mice presented with a pup on two consecutive days showed a significant reduction in latency to attack (Wilcoxon's $Z = 2.201$, $P < 0.05$, mean latency to attack during the first test: 282.8 ± 12.7 sec, during the second: 87.8 ± 41.3 sec). A repetition of this experiment on 16 mice gave similar results ($Z = 3.258$, $P < 0.005$, mean latency to attack during the first test: 83.1 ± 11.7 sec, during the second: 22.2 ± 6.6 sec). Hence, it was preferable to compare directly different substances in simultaneous presentations. In this way, the direction of the first attack could also be recorded, and the variability in absolute values between different groups reduced to a minimum, so that fewer mice were necessary to perform statistical analyses.

Urinary Chemosignals Differentially Affect Resident Behavior. Isolated males exposed to pairs of pups display aggressive behaviors that are consistently modulated by chemical signals. The percentage of mice that received the first attack in each group and the percentage of pups attacked in the first 5 min and at the end of the experiment for each treatment are given (Figure 1). Data on the latency to first attack are summarized (Figure 2) and data from single mice are plotted (Figure 3).

Experiment 1 showed that the number of mice that attacked male-urine-anointed pups after 300 and 900 sec was significantly lower than controls, $\chi_1^2 = 11.56$, $P < 0.001$. The analysis of latencies confirms that attacks on anointed pups were significantly delayed, $Z = 2.911$, $P < 0.005$.

In experiment 2, the number of mice that attacked controls or female smelling pups was not different, $\chi_1^2 = 0.35$, $P = 0.550$. No difference was observed between attack latencies to controls and to female-smelling pups, $Z = 1.249$, $P = 0.211$. This rules out a nonspecific effect of urinary olfactory signals in delaying attacks, and also an effect of cooling pups with liquid or washing away pup odor, since pups anointed with female urine are attacked similarly to controls. Moreover, pups painted with pregnant mice urine were not attacked later than controls, $Z = 0.140$, $P = 0.888$ (data not shown), thus excluding protective effects of pregnant urine. In this case, there was no difference in the number of mice that attacked controls and anointed pups, $\chi_1^2 = 0.16$, $P = 0.683$.

In experiment 3, mice attacked preferentially female rather than male smelling pups, $\chi_1^2 = 5.66$, $P < 0.02$. Pups treated with male urine also were attacked later than those anointed with female urine, $Z = 2.215$, $P < 0.05$.

Which Are the Active Substances in Male Urine? In experiment 4, three pups were presented, two of which were painted with dialyzed or concentrated urine. No significant difference was apparent between conditions ($\chi_2^2 = 1.73$, $P > 0.1$). When comparing each separate condition with controls, however, it was apparent that more mice attacked controls than painted pups ($\chi_1^2 = 11.50$, $P < 0.001$ for concentrated smelling pups vs. controls; $\chi_1^2 = 5.57$, $P < 0.02$ for dialyzed vs. controls, no difference between anointed pups). Pups painted with dialyzed urine and concentrated urine were attacked later than unscented controls, Friedman's ANOVA $\chi^2 = 11.143$, $P < 0.005$, but no difference was apparent between the two painted pups, suggesting that the active substances were present in both urinary fractions. Only the difference between controls and painted groups was statistically significant ($P < 0.02$ when comparing both controls with dialyzed-treated pups, and controls with concentrated urine). This indicates that the effect was induced by small molecules that are present in both dialyzed and concentrated urine fractions.

Experiment 5 showed that more mice attack controls than MUP-painted pups, $\chi_1^2 = 12.14$. Control pups were attacked faster than pups anointed with purified MUPs, $Z = 2.726$, $P < 0.01$. This result together with the previous one suggests

that MUP ligands, bound by the proteins and released in the urine, are sufficient to delay attacks.

In experiment 6, three pups were presented, one control, one painted with MUPs, and one painted with adult male urine. No significant difference was apparent when comparing all conditions ($\chi^2_2 = 4.20$, $P > 0.1$), but when comparing separately each condition with controls, more mice attacked the control pups compared to male urine ($\chi^2_1 = 16.86$, $P < 0.001$) and also compared to

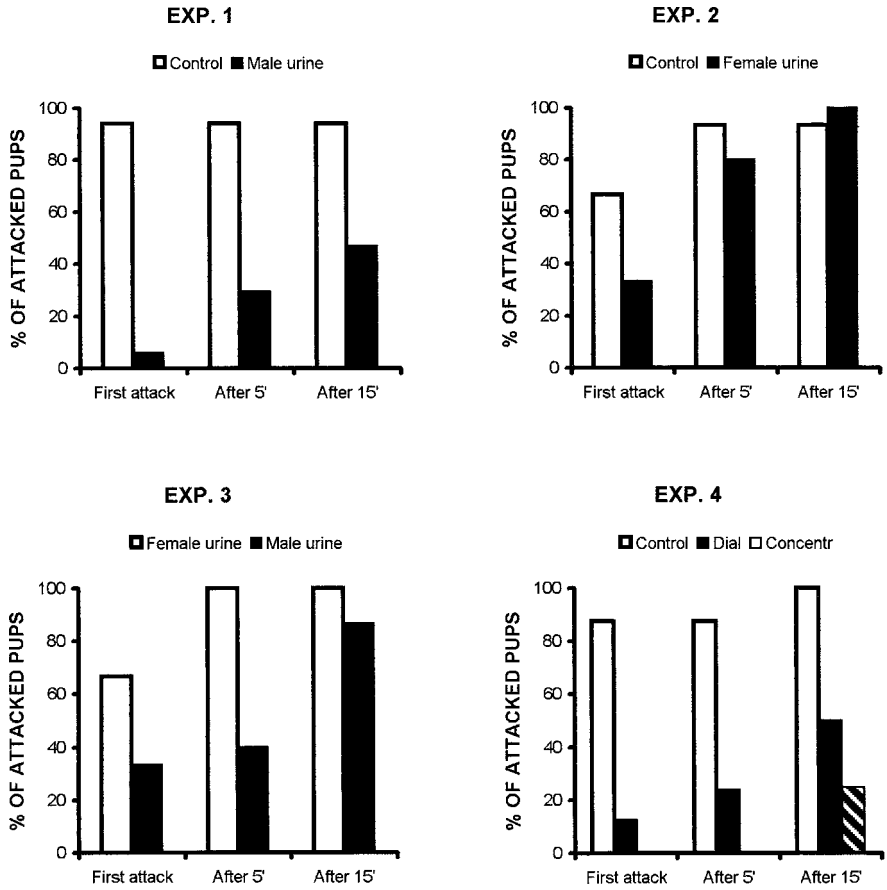

FIG. 1. Percentage of pups that received the first attack in each group, percentage of pups attacked after 5 min in each group, and percentage of pups attacked in each group at the end of the experiment, after 15 min. Dial: urine fraction containing molecules with molecular weight below 3000 Da. Concentr: concentrated urine fraction, containing high molecular weight molecules.

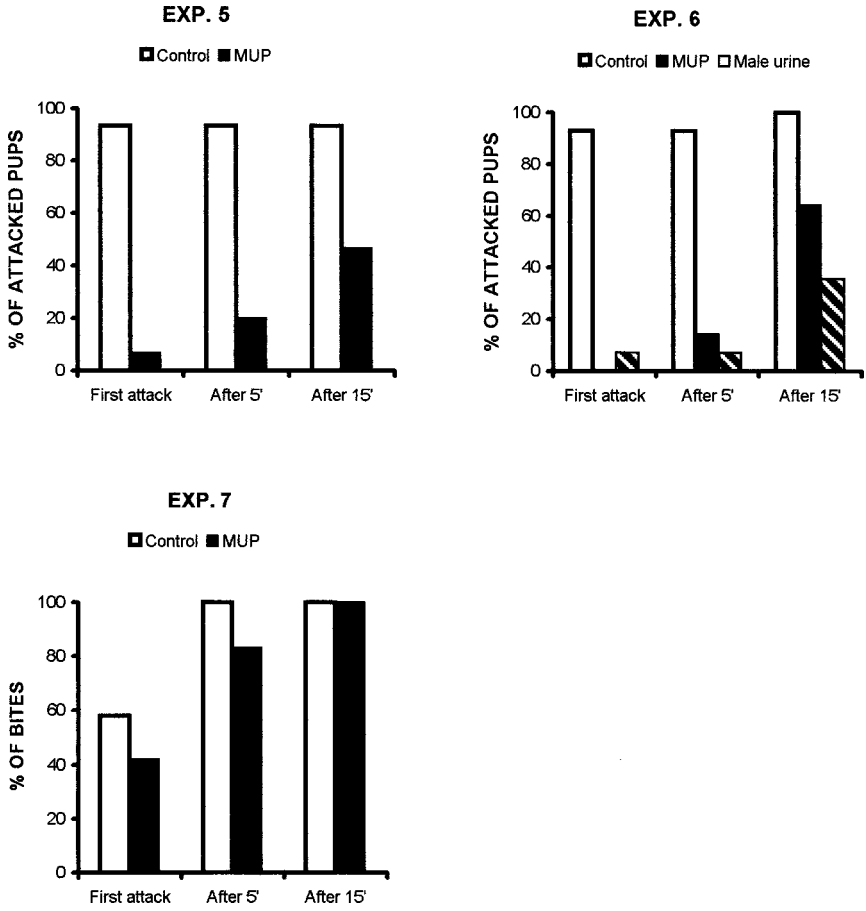

FIG. 1. CONTINUED.

MUPs ($\chi_1^2 = 11.09$, $P < 0.001$), with no difference between painted pups. Both painted pups were attacked later than the control, as shown by latency data, Friedman's ANOVA $\chi^2 = 19.88$, $P < 0.001$, with no difference between the two painted pups. A significant difference is present when comparing the controls with either MUP- or urine-treated pups. This result suggests that in experiment 4 the protective effect of dialyzed fraction is due to the small molecules released by the MUPs that can cross the membrane and are, thus, present in both fractions.

Are the Effects of Male Urine Artifactual? In experiment 7, to test for a potentially nonspecific aversive effect of male chemosignals, food items were anointed with MUPs or not. Under these conditions, the number of mice that bit

control so anointed breadsticks did not differ ($\chi_1^2 = 0.33$, $P = 0.563$). Moreover, there was no difference in the latency to the first sniff, $Z = 0.314$, $P = 0.754$, or in the latency to the first bite on either breadstick, $Z = 1.412$, $P = 0.157$.

DISCUSSION

Chemical signals are known to modulate (and trigger) aggressive interactions in mice. A role in promoting aggression has already been attributed to two

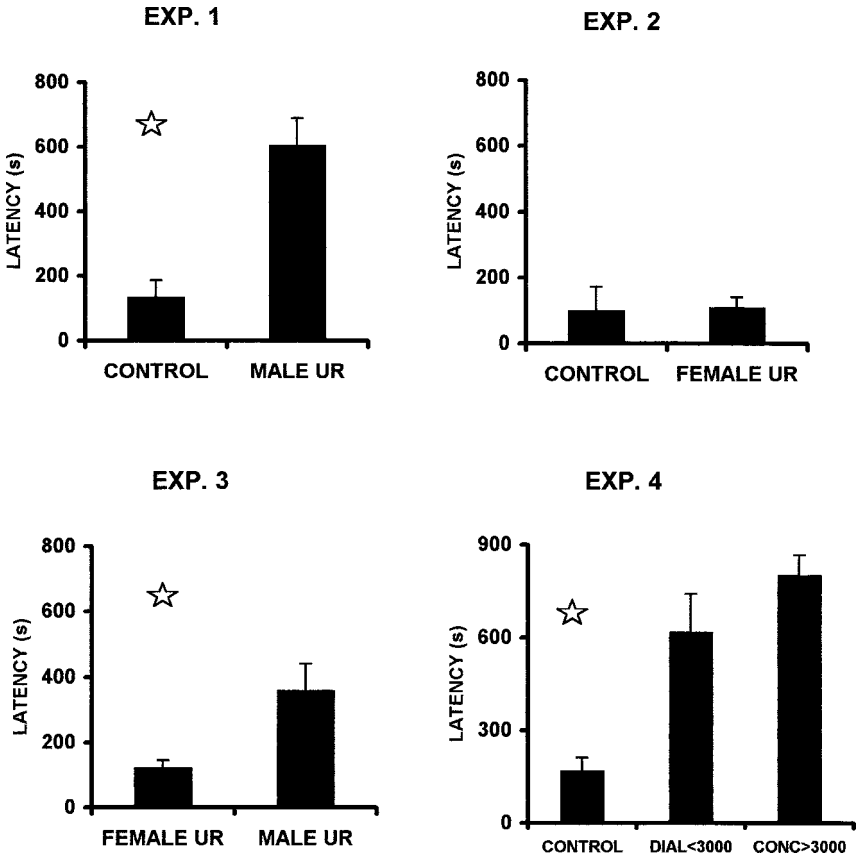

FIG. 2. Latency (in sec) to the first attack (means + SEM) is shown for each experiment. Stars indicate that the control pup is attacked significantly faster than anointed pups. CONTROL: unpainted pups; UR: urine; MUPs: chromatographically purified Major Urinary Proteins; DIAL < 3000: urine fraction containing only low molecular weight molecules (below 3000 Da); CONC > 3000: concentrated urine fraction containing also high molecular weight molecules.

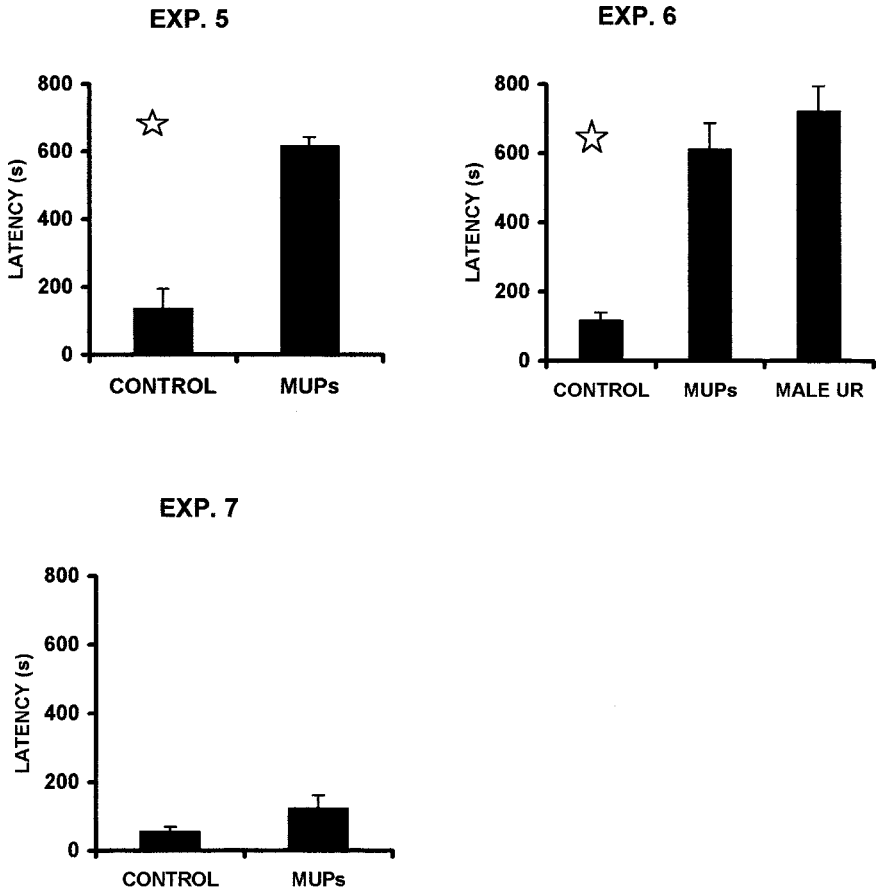

FIG. 2. CONTINUED.

urinary molecules (Novotny et al., 1985). The aim of this work was to investigate whether urinary male chemosignals, already proven to elicit aggression against adult female or castrated mice (Mugford and Nowell, 1970; Novotny et al., 1985; Mucignat-Caretta and Caretta, 1999b), are general aggression signals that also trigger aggressive behavior against newborns.

The present data are not consistent with the notion that male urinary chemosignals act as nonspecific triggers of aggressive behaviors. The same molecules when sprayed on castrated males (Mugford and Nowell, 1970) or on receptive females (Mucignat-Caretta and Caretta, 1999b), elicit aggression, but in contrast inhibit it when painted on pups. Moreover, urine alone is not sufficient to delay infanticide, as shown with female urine in experiment 2. Our data indicate that whole male urine protects pups from immediate attacks (experiments 1 and 3), that this effect

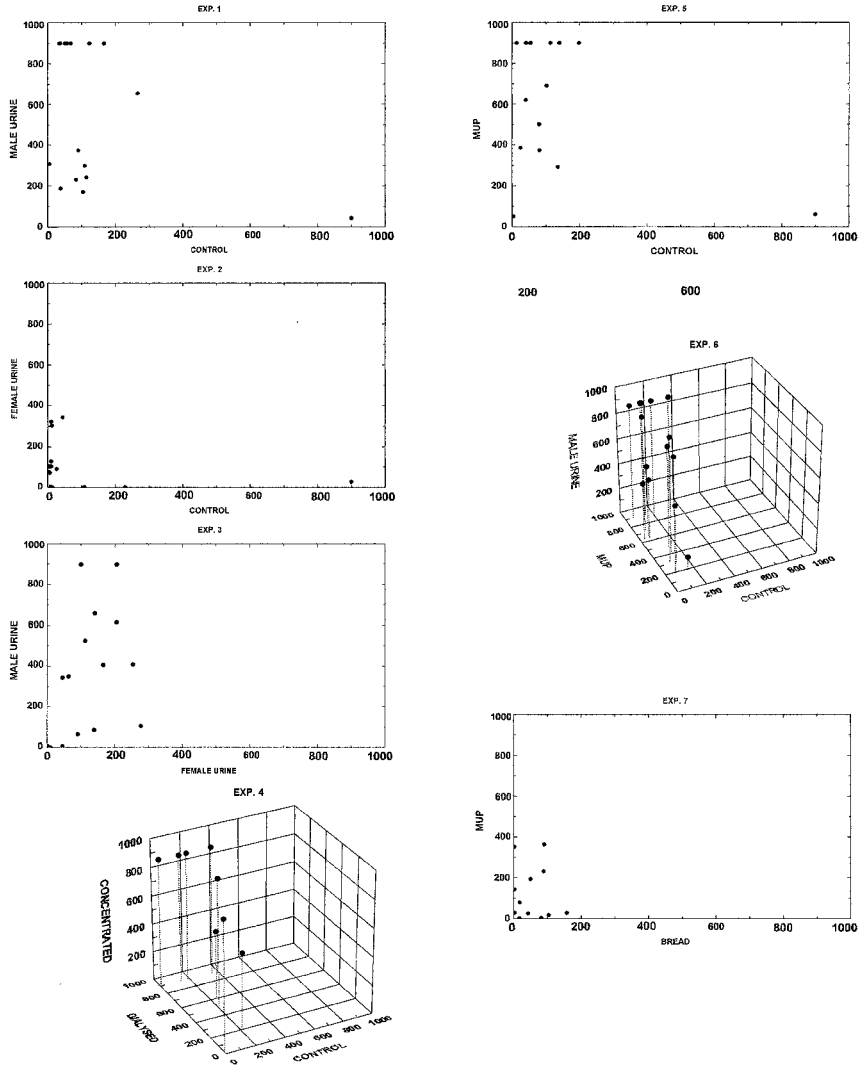

FIG. 3. Latencies of individual mice are shown for each experiment. The latency to attack the control pup is plotted against the latency to attack the painted pup. When three pups are presented, a three-dimensional space is used. Each point represents one mouse, the projection of the point on the relevant axis is the latency (in sec).

is due to volatile molecules, and that these active molecules are the ones bound by the MUPs (experiments 4, 5, and 6). MUP-borne molecules appear to act as flags of an adult male presence, but the signal has to be validated with other information and interpreted in different situations before triggering or inhibiting a specific aggressive behavior. We suggest that a male mouse perceives male urinary chemicals as fundamental cues to the presence of an adult opponent male. In such cases of competitive aggressive behavior (Mugford and Nowell, 1970), these signals can mislead the resident male to preventive attacks against adult females or castrated males sprayed with male urine. In the presence of an anointed pup, however, a striking contrast arises with addition sensory information coming from the pup, visual, acoustic, or ultrasonic (Blumberg and Sokoloff, 2001). These same chemical signals do not cause the adult mouse to confuse a pup with an opponent, and surprisingly they delay the onset of aggression. Under these circumstances male urine may signal to others the presence of an adult male (the father?) that is potentially able to defend the newborn.

Thus, the idea of aggression-promoting pheromones acting in a reflex-like way can be questioned. Most male chemosignals are commonly found in the environment. They could cause useless aggression displays toward meaningless stimuli, and ultimetely decrease fitness. Presumably, mice detect a mixture of MUPs and their ligands and perceive them as a holistic, Gestalt-like (Metzger, 1941) signal and interpret it together with other contextual cues.

The same chemicals have been shown to attract females and repel males in open field environments (Mucignat-Caretta et al., 1998), but diminish aversion of males toward hostile environments (Mucignat-Caretta and Caretta, 1999a) and modulate female exploratory behavior (Mucignat-Caretta, 2002). Additionaly, these chemical stimuli induce aggression toward receptive females (Mucignat-Caretta and Caretta, 1999b), but delay attacks on pups.

Distinct types of aggressive behaviors have been reported (Ferrari et al., 1996), and the loss of different subtypes of vomeronasal receptors differentially affects different types of aggression (Del Punta et al., 2002). These results indicate that aggressive behaviors are variably modulated by chemical cues together with signals coming from other sensory inputs; the behavioral effect is, thus, related to the integration of the various sensory information in different contexts.

MUPs and their ligands are only a minor fraction of urinary constituents, but they consistently modulate the behavior of a recipient mouse. It is possible that this modulation is not due to single molecules but to the “maleness blend” (Cavaggioni and Mucignat-Caretta, 2000; Cavaggioni et al., 2001). This is an intriguing possibility, if we accept that the perceptual quality of olfactory mixtures is different from the sensation evoked by single molecules, either from subjective reports in humans or considering the activation pattern of glomeruli in the olfactory bulbs (Rubin and Katz, 1999).

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A STINGLESS BEE (*Melipona seminigra*) MARKS FOOD SOURCES WITH A PHEROMONE FROM ITS CLAW RETRACTOR TENDONS

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Abstract—By depositing scent marks on flowers, bees reduce both the search time and the time spent with the handling of nonrewarding flowers. They thereby improve the efficiency of foraging. Whereas in honey bees the source of these scent marks is unknown, it is assumed to be the tarsal glands in bumble bees. According to histological studies, however, the tarsal glands lack any openings to the outside. Foragers of the stingless bee *Melipona seminigra* have previously been shown to deposit an attractant pheromone at sugar solution feeders, which is secreted at the tips of their tarsi. Here we show that the claw retractor tendons have specialized glandular epithelia within the femur and tibia of all legs that produce this pheromone. The secretion accumulates within the hollow tendon, which also serves as the duct to the outside, and is released from an opening at the base of the unguitractor plate. In choice experiments, *M. seminigra* was attracted by feeders baited with pentane extracts of the claw retractor tendons in the same way as it was attracted by feeders previously scent marked by foragers. Our results resolve the seeming contradiction between the importance of foot print secretions and the lack of openings of the tarsal glands.

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Key Words—Hymenoptera, Apidae, Meliponini, stingless bee, *Melipona*, tendon gland, food source marking, pheromone, attractant, hydrocarbons.

INTRODUCTION

Honey bees (Apidae, Apini), bumble bees (Apidae, Bombini), and stingless bees (Apidae, Meliponini) use repellent scent marks to keep foragers from visiting recently depleted flowers (Núñez, 1967; Giurfa and Núñez, 1992; Giurfa, 1993; Goulson et al., 1998, 2001; Stout et al., 1998; Williams, 1998; Stout and Goulson, 2001) as well as attractant substances to mark rich food sources (Ferguson and Free, 1979; Cameron, 1981; Free and Williams, 1983; Schmitt and Bertsch, 1990; Villa and Weiss, 1990; Nieh, 1998; Aguilar and Sommeijer, 2001; Hrncir et al., 2004; Nieh et al., 2003; Schmidt et al., 2003). The bees thereby improve the efficiency of food gathering by reducing both the time spent with nonrewarding flowers and the search for rewarding flowers (Wetherwax, 1986; Kato, 1988; Schmitt and Bertsch, 1990; Giurfa and Núñez, 1992).

The stingless bee *Melipona seminigra* marks rich food sources with attractant scent marks (Hrncir et al., 2004). After about 40 visits of one or more foragers at a sugar solution feeder, newly arriving bees prefer this food source to a clean but otherwise identical control feeder. The scent marks remain effective for at least 2 hr and were shown to be left on the substrate as "foot prints." When the bees' leg tips were covered with nail polish, thereby sealing the openings of excretory ducts of glands within the legs, *Melipona* was no longer able to mark a feeder even after 80 visits (Hrncir et al., 2004).

In honey bees, the origin of the scent marks left at food sources by foragers remains unresolved (Free, 1987; Winston, 1987). Foot print secretions are known to be used to mark the entrance of the hive to help returning bees find their way home (Butler et al., 1969). Bumble bees are assumed to deposit both their repellent and their attractant scent marks at food sources as foot prints. The tarsal glands within the fifth tarsomeres of their legs were considered the most obvious candidate glands producing the foot print substances (Schmitt et al., 1991; Stout et al., 1998; Goulson et al., 2000). In histological studies, however, openings of the tarsal glands to the outside could not be found in bumble bees (Pouvreau, 1991) or honey bees (Lensky et al., 1985; Federle et al., 2001), nor in *M. seminigra* (Jarau, unpublished) or other stingless bees (Cruz-Landim et al., 1998).

The present study was undertaken (i) to look for other possible glandular origins of the foot print substances used by *M. seminigra* to scent mark food sources, (ii) to examine the effect of gland extracts on the bees' behavior, and (iii) to analyze the chemical composition of scent marks and of gland extracts.

METHODS AND MATERIALS

Bee Colonies. We used three colonies of *M. seminigra* FRIESE 1903 (Hymenoptera, Apidae, Meliponini) that were collected in Nova Xavantina–MT, Brazil, and housed in wooden boxes inside the laboratory, connected to the outside by plastic tubes in the wall of the building. The experiments were carried out on the Ribeirão Preto—Campus of the University of São Paulo.

Histology and SEM. Legs of foraging workers were fixed in Dubosq-Brasil solution for 72 hr, washed in 70% ethanol, dehydrated in a series of alcohols with increasing concentration, and embedded in Spurr's resin. Serial sections 5–8 μm thick were stained with toluidine blue. For scanning electron microscopy, legs were stored in 70% ethanol, dehydrated in a series of alcohols with increasing concentration, placed in acetone, and finally dried using hexamethyldisilazane. Preparations were mounted on stubs, sputter coated (Agar 103) with gold for 300 sec, and examined in a Jeol JSM-35CF scanning electron microscope.

Bioassays. Gland extracts were prepared by dissecting the entire claw retractor tendons with their glandular tissues from the legs of foraging workers. To avoid contamination with material from the tarsal glands, the fifth tarsal segments were removed prior to cutting the legs and taking out the tendons. Two extracts were prepared by placing the 18 tendons from three bees in 1200 μl pentane for 24 hr each. Four hundred microliters of solution therefore corresponded to one bee equivalent (six tendons). For testing the bees' behavioral reaction to different concentrations of the gland extracts, 8, 40, and 200 μl of the stock solutions corresponding to 0.02, 0.1, and 0.5 bee equivalents were used, respectively.

Bees were trained to collect unscented sugar solution (50% weight on weight) *ad libitum* at a training feeder 20 m northwest of the hives between 10:00 and 11:30 on days when no choice experiments were performed. The feeders used for the bioassays were small plastic cups containing 50% unscented sugar solution on plastic discs (65 mm in diam). The distance between the test feeder (baited with gland extract at the beginning of the experiment) and the control feeder (same amount of pure pentane) measured 20 cm consistently. To avoid side bias, the positions of the two feeders were exchanged every 5 min. When the setup was mounted at 10:00 at the training site, a few drops of sugar solution were injected into the nest entrances at the same time in order to stimulate the foragers. The bees quickly started to visit the known feeding site. For 40 min, the number of bees that landed and extended their proboscises into the sugar solution on either the test feeder or the control feeder was recorded. All bees were captured on their first visit to one of the feeders, marked, and released at the end of the respective experiment only. In order to avoid individual bees being tested more than once, only those captured during a given experiment and not already marked in preceding experiments were included in the analysis. Control experiments followed the same procedure, but the bees had to choose between two identical clean feeders. For

each bee equivalent tested and for the control series, six experiments were carried out. During all 24 experiments ($N = 24$), a total of 465 individual bees (control: $N = 137$; 0.02 bee equivalents: $N = 115$; 0.1 bee equivalents: $N = 131$; 0.5 bee equivalents: $N = 82$) were tested.

Data Analyses. We compared the median percentage of bees choosing the test feeder in the choice experiments and the percentage of bees choosing one (defined prior to the experiment) of the two identical control feeders (during the control experiments) using Mann–Whitney *U* tests followed by the sequential Bonferroni procedure (overall $P < 0.05$) in order to control for tablewide type I errors (Rice, 1989).

Chemical Analyses. Scent marks left on artificial flowers were collected using four cleaned (rinsed with ethanol, acetone, and pentane; heated 3 hr in an oven at 230˚C) Teflon® discs (Ø = 18 mm) as "petals." The bees landed on these discs and took up sugar solution (50% weight on weight) from a small plastic cup that was continuously refilled by means of a syringe connected to its bottom. For chemical analyses, washings of 12 Teflon® discs (pooled), which contained the substances left by a total of 2000 bee visits, were used. Extracts of the fifth tarsomeres with the tarsal glands and of the dissected claw retractor tendons with the glandular epithelium (dissected as described for the bioassays) were prepared by extracting the respective parts of the legs (150 tarsomeres and 150 tendons) in 1 ml pentane for 24 hr. All samples were analyzed using a combined Fisons Instruments GC 8000 series/MD 800 mass spectrometer (carrier gas: helium; column: 30 m × 0.25 mm fused silica coated with DB5-MS, 0.25 μm thickness; electron impact 70 eV). The temperature was initially kept at 60˚C for 5 min, then increased by 10˚C min^{-1} to 300°C, and kept at this temperature for 30 min. Double bond positions in alkenes were determined by investigation of the corresponding dimethyl disulfide adducts (Buser et al., 1983), while geometries of double bonds were assigned on the basis of reference substances, synthesized by standard laboratory methods.

RESULTS

Glandular Structures. In the legs of *Melipona*, a claw retractor tendon runs from the femur through the tibia and tarsus and connects to the unguitractor plate at the base of the pretarsus. The claw retractor muscle is divided into a femoral and a tibial part (Figure 1a). The epidermis covering the tendon partly forms a glandular epithelium within the femur and the tibia (Figure 1b and c). This epithelium is well developed in all legs. The tendon itself forms a hollow cuticular tube along its entire length and opens to the outside at the base of the unguitractor plate (Figure 1d). Inside the fifth tarsomere, the claw retractor tendon crosses the epithelium of the tarsal gland, but there is no connection between the lumina of the tarsal gland and the tendon gland, respectively. The secretion produced by the

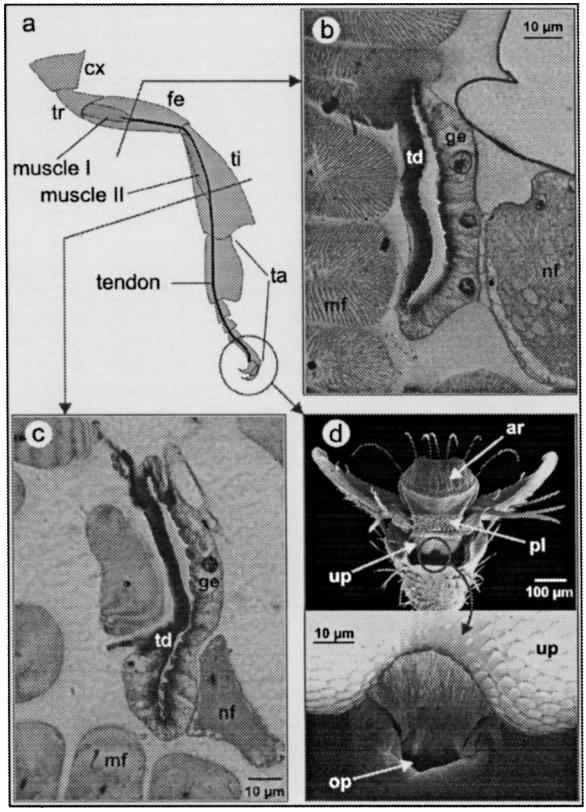

FIG. 1. Tendon gland. (a) Diagram of a metathoracic leg illustrating the location of the tendon and its associated muscles. (b and c) Cross sections through the tendon gland in femur and tibia, respectively; note the cuticular part of the tendon (denoted by "td"), the large cells of the glandular epithelium (denoted by "ge"), and the lumen inside the tendon. (d) Ventral view of the fifth tarsomere and the pretarsus (top) and of an enlargement (bottom) of the opening (denoted by "op") of the tendon at the base of the unguitractor plate (denoted by "up"). Further abbreviations: ar—arolium; cx—coxa; fe—femur; mf—muscle fibres; nf—nerve fibres; pl—planta; ta—tarsus; ti—tibia; tr—trochanter.

glandular epithelium accumulates inside the tendon, particularly in the enlarged sections within the femur and the tibia. Very likely, it is pressed through the opening when the claw retractor muscles contract and the tendon diameter decreases due to increased tension.

Bioassays. The choice experiments show that extracts exclusively taken from the tendon glands described above are in fact an attractant and that the attractiveness of a feeder baited with it is dose dependent (Figure 2a). The percentage of

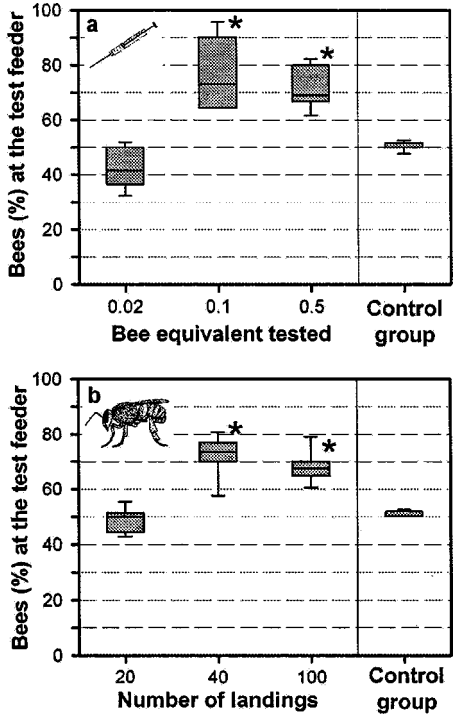

FIG. 2. Percentage of bees choosing a test feeder. (a) Choice experiments testing the effect of tendon gland extracts. (b) For comparison, results from Hrncir et al. (2004) for feeders visited by 20, 40, or 100 foragers prior to testing are shown. The number of bees choosing the test feeder depends on the concentration of the attractant pheromone deposited on it (i.e., bee equivalents in (a) and accumulation of scent marks in (b)). The boxes represent the medians with 1st and 3rd quartiles, the bars below and above a box indicate the 10th and 90th percentile, respectively. Asterisks (*) indicate experiments with significantly more bees choosing the test feeder than one of the two feeders in the respective control experiments (Mann–Whitney U tests and sequential Bonferroni method; $P = 0.002$ in (a) and $P = 0.004$ in (b)).

bees choosing the feeders baited with 0.02 bee equivalents was not significantly different from the control experiments ($U = 7.5$, $N_1 = N_2 = 6$, $P = 0.093$). When feeders were baited with 0.1 and 0.5 bee equivalents, however, a significantly larger percentage of bees landed on the test feeder as compared to the control experiments (for both comparisons: $U = 0$, $N_1 = N_2 = 6$, $P = 0.002$). The percentages of bees choosing the baited feeder in the experiments with 0.1 and 0.5 bee equivalents, respectively, did not differ significantly from each other ($U = 14$, $N_1 = N_2 = 6$, $P = 0.589$). To compare these results with data for bees choosing between a clean

feeder and a feeder visited by 20, 40, or 100 foragers prior to testing, results from Hrncir et al. (2004) are shown in Figure 2b.

Chemical Analyses. According to chemical analyses, the foot prints left by *M. seminigra* foragers at food sources contain a complex mixture mainly of hydrocarbons (Table 1). The same substances are also found in the tendon glands and in the extracts of the last tarsal segments, which contain the tarsal glands. In addition to these substances, another 41 compounds could be identified in the tendon gland and the tarsal extracts (a number of methylalkanes and wax-type esters in both extracts; additional fatty acids and fatty acid ethyl esters in the tarsal glands; Table 1).

DISCUSSION

Despite the many studies demonstrating the importance of repellent and attractive scent marks at food sources (see Introduction), knowledge about the origin of these scent marks is scarce. Foot print substances are at least used by bumble bees (Schmitt et al., 1991; Stout et al., 1998; Goulson et al., 2000) and by *M. seminigra* (Hrncir et al., 2004). Considering the lack of openings of the tarsal glands to the outside (Lensky et al., 1985; Pouvreau, 1991; Cruz-Landim et al., 1998; Federle et al., 2001; Jarau, unpublished), secretion of the respective pheromones by them is unlikely. The tendon glands of *M. seminigra* described in the present study resolve the contradiction between the known importance of foot print secretions on the one hand and the lack of openings of the tarsal glands on the other.

Attractiveness of Tendon Gland Extracts. According to our choice experiments, the attractive pheromone left by the foragers of *M. seminigra* at the food source is produced by the glandular epithelium of their claw retractor tendons. Interestingly, the dose dependence of the attractiveness now found in the bioassays supports results of our earlier study (Hrncir et al., 2004). In this study, the ability to choose a sugar solution feeder scent marked by foragers was shown to depend on the number of the preceding bee visits (Figure 2b). It reached a maximum after 40 forager landings and decreased at still higher numbers. Likewise, the attractiveness of the scent marks reached a maximum at a pheromone concentration corresponding to 0.1 bee equivalent and decreased again at a higher concentration. Thus, at still higher concentrations, the pheromone may also function as a repellent signal indicating flowers that have already been depleted by many visits (many scent marks accumulated). Experiments with bumble bees showing corresponding effects of synthetic mixtures of hydrocarbons previously identified in scent marks (Schmitt et al., 1991) support this assumption. It has long been known that in social insects the same pheromone can release different behaviors at different concentrations (Wilson, 1965).

TABLE 1. SUBSTANCES IDENTIFIED FROM FOOTPRINTS LEFT AT FOOD SOURCES AND FROM EXTRACTS OF THE TENDON GLANDS AND THE FIFTH TARSAL SEGMENTS, RESPECTIVELY, OF FORAGERS OF *Melipona seminigra*[a]

Substance	Foot prints	Tendon glands	5th tarsal segment
Alkanes			
Heptadecane	++	+	+
Nonadecane		+	++
Eicosane		+	
Heneicosane	++	+	+
Docosane	++		++
Tricosane	+++	++	+
Tetracosane	++	++	++
Pentacosane	++++	++++	+++
Hexacosane	+++	++	+
Heptacosane	++++	+++	+++
Octacosane	+++	+	+
Nonacosane	+++	++	++
Triacontane	+++	+	
Hentriacontane	+++	++	++
Tritriacontane		+	
Alkenes			
8-(*Z*)-Heptadecene		+	
9-(*Z*)-Nonadecene	+	+	+
7-(*Z*)-Nonadecene		+	
9-(*Z*)-Tricosene	++	+	+
7-(*Z*)-Tricosene	++	++	++
7-(*Z*)-Tetracosene		++	++
9-(*Z*)-Pentacosene	++	++	++
7-(*Z*)-Pentacosene	++++	++++	++++
9-(*Z*)-Hexacosene		+	
7-(*Z*)-Hexacosene		++	++
9-(*Z*)-Heptacosene	+++	++	+++
7-(*Z*)-Heptacosene	++++	++++	+++
9-(*Z*)-Nonacosene			++
7-(*Z*)-Nonacosene	+++	+++	+++
Methyl alkanes			
11-Methyltricosane		+	+
9-Methyltricosane		+	+
7-Methyltricosane		+	+
5-Methyltricosane		+	++
3-Methyltricosane	++	++	++
13-Methylpentacosane		+	++
11-Methylpentacosane		+	++
9-Methylpentacosane		+	++
7-Methylpentacosane		+	+
5-Methylpentacosane		++	+
3-Methylpentacosane		++	++

Continued

TABLE 1. CONTINUED

Substance	Foot prints	Tendon glands	5th tarsal segment
13-Methylheptacosane		+	++
11-Methylheptacosane		+	++
5-Methylheptacosane		+	+
3-Methylheptacosane		+	+
3-Methylnonacosane		+	
Aldehyde			
Octadecanal	+++		++
Acids			
Tetradecanoic acid			++
Hexadecenoic acid			++
Hexadecanoic acid			++
Octadecanoic acid			++
Esters			
Ethyl hexadecanoate			++
Ethyl linoleate			++
Ethyl oleate			+++
Ethyl linolenoate			+++
Tetradecyl tetradecanoate		+	+
Dodecyl hexadecanoate		+	+
Dodecyl octadecenoate		+	
Tetradecenyl hexadecenoate		+	++
Tetradecyl hexadecanoate		++	++
Tetradecenyl octadecenoate		++	++
Hexadecenyl hexadecenoate		+	
Hexadecyl hexadecanoate		+	++
Hexadecenyl octadecenoate		++	++

[a] (+) <0.1%; (++) 0.1–0.9%; (+++) 1.0–9.9%; (++++) ≥10%.

Tendon Glands. The development of parts of the epidermis of the claw retractor tendons into pheromone producing glands is an intriguing example of the adaptation of a structure already serving a particular function to a different one. To the best of our knowledge, our study is the first to demonstrate the behavioral significance of this gland in a bee. Importantly, however, ants of the genus *Crematogaster* lay scent trails using a pheromone produced in their metathoracic legs (Fletcher and Brand, 1968). The glands involved were described as a reservoir lined by cuticle and surrounded by a monolayered epithelium inside the tibia, with a hollow tendon guiding the secretions to the pretarsal opening (Billen, 1984). Although not explicitly stated by the author, the tibial gland in *Crematogaster* most likely represents a specialized region of the claw retractor tendon similar to that now found in the stingless bee. In contrast to the glands of *M. seminigra*, however, the glands of *Crematogaster* were only found in the tibiae and were far less

developed in the first and second legs than in the hind legs. In the queens of five species of stingless bees, sacculiform glands formed by a fold of the epidermis covering the apodeme of the femoral muscles have been described recently (Cruz-Landim et al., 1998). Openings of these glands could not be found, however, and their function remains unknown. The question of how widespread (in terms of both phylogeny and behavioral context) the use of tendon glands is within the social Hymenoptera (or in insects in general) can only be answered by extending the study to other groups. Our assumption that the foot print substances are secreted by tendon glands instead of tarsal glands is strongly supported by recent findings in honey bees and weaver ants, which demonstrate that the liquid content of the tarsal gland reservoir is not released to the outside but serves to fill the arolium in order to increase adhesion to the substrate during walking (Federle et al., 2001).

Scent Marks and Gland Contents. The scent marks left at food sources by *M. seminigra* mainly consist of hydrocarbons. Most of these hydrocarbons are also contained in tarsal gland extracts and foot print substances of the bumble bee, *Bombus terrestris* (Schmitt et al., 1991), where they serve as attractant scent marks at sugar solution feeders like in *Melipona*. Goulson et al. (2000) found some of these hydrocarbons in tarsal extracts of *B. terrestris*, *B. pascuorum*, and *B. lapidarius* as well. These authors, however, reported a repellent effect of tarsal extracts on foraging bumble bees when applied to flowers. For *M. seminigra*, no data on the effect of foot prints or of tendon gland extracts on bees foraging at natural food sources are available yet. Also, it is not known yet whether the hydrocarbons themselves are the active compounds in the foot prints of *M. seminigra* or rather serve as solvents for other substances.

As our analyses show, the chemical composition of the tendon gland extracts is similar to that of the last tarsal segments containing the tarsal glands in *M. seminigra*. Dahl (1885) interpreted the tarsal glands of the honey bee as saclike foldings of the epidermis of the most distal parts of the claw retractor tendons. A common ontogenetic origin of tendon glands and tarsal glands may well explain the chemical similarity of their products. It has also to be taken into account, however, that it is difficult to completely exclude small pieces of the tendons and their secretions when preparing extracts of the tarsal glands.

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NEW TYPE OF SESIIDAE SEX PHEROMONE IDENTIFIED FROM THE HORNET MOTH *Sesia apiformis*

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Abstract—Two components of the female-produced sex pheromone of the hornet moth, *Sesia apiformis*, were identified as (3*Z*,13*Z*)-octadeca-3,13-dien-1-ol (3*Z*,13*Z*-18:OH) and (2*E*,13*Z*)-octadeca-2,13-dienal (2*E*,13*Z*-18:Al), a pheromone structure new in Sesiidae. Pooled gland extracts showed the two major compounds in a proportion of *ca.* 2:3, while SPME-investigations on single calling females revealed a ratio of *ca.* 1:7. Although the single compounds were not attractive, a 2:3 mixture proved to be highly active towards males in field tests. Small amounts of (2*E*,13*Z*)-octadecadienol (2*E*,13*Z*-18:OH) were found in the sex pheromone gland of females, however, the biological significance of the compound remains unclear. Methyl sulfide was found to readily react with 2-alkenals, providing an effective new method for the characterization of this type of compound upon GC/MS. The derivatives, 1,1,3-tris(methylthio)alkanes, are the products of the addition of methyl sulfide to the double bond and the transformation of the carbonyl group into the corresponding bis(methylthio)acetal. The mass spectra of these compounds are characterized by diagnostic signals at *m*/*z* 107 and/or *m*/*z* 121. These fragments represent the first carbon unit or the first two carbon units of the derivative, respectively. The parent signal in the spectra of thiomethyl derivatives of 2-alkenals showing no other double bonds is represented by *m*/*z* $M^+ - 121$, formed upon loss of the first two carbon units. By employing a solution of methyl sulfide in dimethyl sulfide, the double bond positions in 2*E*,13*Z*-18:Al could be fully characterized by GC/MS.

Key Words—Pheromone, Lepidoptera, Sesiidae, field trapping, 2-alkenal, methyl sulphide.

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INTRODUCTION

The hornet moth, *Sesia apiformis* (Cl.) (Lepidoptera: Sesiidae), is an important pest of poplar (*Populus* spp.) throughout the whole holarctic region (Postern, 1962; Fibiger and Kristensen, 1974; Heppner and Duckworth, 1981; Kolomoets, 1995). The caterpillars' damage to roots and the lower trunk results in withering or death of the tree. In addition, the caterpillars' galleries provide favorable conditions for fungal infections. The cryptic nature of larval development as well as the urban site of infestations render the control of this pest complicated and conventional application of insecticides ineffective. The identification and synthesis of the sex pheromone may open an opportunity to establish an effective monitoring system and to develop new control methods.

The composition of sex pheromones and attractants among Sesiidae is highly conserved, as to date only the *E*,*Z*- and *Z*,*Z*-isomers of 3,13- and 2,13-octadecadienol as well as the corresponding acetates are known (Arn et al., 2002). Extensive field screening has shown these compounds attract many clearwing species (Szöcs et al., 1989, Būda et al., 1993; Mozūraitis et al., 1999; Karalius et al., 2001), yet the natural composition of pheromone blends of European *Sesia* species have not been established.

Analytical investigations of the pheromone system of *S. apiformis* were initiated two decades ago, with the identification of (3*Z*,13*Z*)-octadeca-3,13-dien-1-ol (3*Z*,13*Z*-18:OH) from extracts of the female sex pheromone gland (Kovalev et al., 1988). However, as this compound was not attractive to *S. apiformis* males (Būda, unpublished), its role as a component of the female sex pheromone remained uncertain.

In this paper we report on the structure elucidation of the *S. apiformis* female sex pheromone as well as field results of tests with synthetic compounds proving the activity of the identified compounds. Moreover, we describe a method to produce derivatives of 2-alkenals by the reaction with methyl sulfide to unambiguously locate the double bond position.

METHODS AND MATERIALS

Insects. S. apiformis cocoons were collected in spring from damaged poplar (*Populus* spp.) in two regions: Vilnius, Lithuania, and Donetsk, Ukraine. Cocoons were kept in moistened sawdust at 20–23°C under natural light conditions (approximately 12L:12D) until adults emerged.

Collection and Analysis of Compounds Released by Individual Calling Females. The solid phase micro extraction (SPME) technique (Borg-Karlson and Mozūraitis, 1996) was used for collecting compounds released from individual calling females. Before the collection periods, the routine cleaning of the SPME

fiber (100 mm polydimethylsiloxane, Supelco, Bellefonte, PA) was done at 225°C for 10 min in a GC injector, splitless mode to ensure the fiber was clean. Subsequently, the tip of the syringe was placed a few millimeters from the protruded abdominal gland of a calling female, who was sitting inside a 500 ml glass tube. The sorption was conducted at 16–18°C for 2–4 hr during the calling period.

The volatiles collected from females during their calling and noncalling periods were compared by coupled gas chromatography mass spectrometry (GC–MS), and only those released exclusively by signalling moths were analyzed. The compounds selected for analysis were identified by comparison of their mass spectra and GC-retention times with corresponding data of synthetic standards. Synthetic alcohols used as GC–MS standards were obtained from Flora Co, Tartu, Estonia.

Volatile compounds collected from calling females were analyzed by using a Finnigan SSQ 7000 GC–MS system (Termo-Finnigan, San Jose, CA), including a Varian 3400 GC (Varian Inc, Palo Alto, CA). A DB-5 and a DB-wax capillary column (30 m; i.d. 0.25 mm; film thickness 0.25 μm; J & W Scientific, Folsom, CA) were used with a temperature program of 80°C (1 min), increased by 20°C/min to 150°C, then by 1°C/min up to 200°C, and thereafter maintained constant at 200°C for 20 min. Both columns were used with the same temperature program. The split/splitless injector temperature was 200°C, and the splitless period was 30 sec. Helium was used as the carrier gas with an inlet pressure of 5 psi. EI-mass spectra were obtained at 70 eV with the ion source at 150°C. When the sample had been injected, the desorption of natural volatiles and synthetic reference compounds from the SPME fiber was checked by subsequent injection in a split/splitless injector of a second GC for 30 sec of splitless time at an injector temperature of 225°C. It was found that total desorption of the compounds had occurred during the first injection.

Extraction and Structure Elucidation of Female-Produced Volatiles. Individual females were freeze killed when they started calling. The pheromone gland was immediately excised and extracted with 40 μl of pentane for 2 min. This extraction procedure was repeated twice for each gland, and subsequently the extracts of 10–20 females were combined, concentrated (Klimetzek et al., 1989), and kept at −20°C until analyzed. In total, extracts of 46 and 50 females were prepared from Vilnius and Donetsk populations, respectively.

For structure elucidation, coupled GC–MS was performed with a VG 70-250 double focussing mass spectrometer (Vacuum Generators, Manchester, UK) linked to an HP 5890 gas chromatograph (Hewlett-Packard). EI-mass spectra were run at 70 eV. Separation of volatiles was carried out by employing a fused silica capillary DB-1 (25 m; i.d. 0.25 mm; film thickness 0.25 μm; J & W Scientific, Folsom, CA) under temperature programming at 4°C/min from 60 to 300°C. Determination of double bond positions in the natural products was carried out by the reaction of crude extracts with dimethyl disulfide (Buser et al., 1983). In a second set of

methylthiolation experiments, a solution of methyl sulfide in dimethyl disulfide was employed under the same conditions.

Synthesis. Structural proof of synthetic compounds was obtained by NMR-spectroscopy and mass spectrometry. NMR-spectra were recorded with a Bruker AMX 400 (Karlsruhe, Germany); chemical shifts (δ) are given in parts per million (ppm) relative to tetramethylsilane (internal standard 0 ppm). The synthesis of 3*Z*,13*Z*-18:OH was similar to that of Hoskovec et al. (1990), while (2*E*,13*Z*)-octadeca-2,13-dien-1-ol (2*E*,13*Z*-18:OH) was prepared according to Ramiandrasoa and Descoins (1989). The oxidation of 2*E*,13*Z*-18:OH to (2*E*,13*Z*)-octadeca-2,13-dienal (2*E*,13*Z*-18:Al) followed the protocol of Takikawa et al. (1997); other 2-alkenals were obtained correspondingly. NMR-data of the samples were in accord with those described in the literature.

At -10°C, 50 mg of 2*E*-decenal (Aldrich) were added to a solution of 100 mg methyl sulfide (Aldrich) in 1 ml diethyl ether (Aldrich) (obtained by bubbling methyl sulfide through diethyl ether at -10°C). After addition of a catalytic amount of iodine (10 μl of a solution of 60 mg iodine in 1 ml diethyl ether), the mixture was kept for 48 hr at 60°C in a gas-tight screw cap vial. After cooling to room temperature, 0.5 ml pentane and a solution of 25 mg sodium thiosulfate was added in 0.5 ml water (after some shaking the initially brown solution turned almost colorless). After adding solid sodium chloride until some remained undissolved, the organic layer was removed and concentrated. The crude product was purified by column chromatography (60 g Merck Silica 60, 230–400 mesh, 0.5 bar, 5–30% ethyl acetate in hexane) yielding 36 mg (40%) 1,1,3-tris-methylthiodecane as a slightly brownish oil. By using dimethyl disulfide as the solvent, the yield was almost quantitative.

^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 0.86 (t, 3H, J = 6.6 Hz, 10-H); 1.25–1.56 (m, 12H, 4-H to 9-H); 1.85 (dd, 1H, J = 6.6 Hz/1.52 Hz, 2-Ha); 1.87 (dd, 1H, J = 6.12/1.56 Hz, 2-Hb); 1.99 (s, 3H, 3-CH_3); 2.06 (s, 3H) and 2.10 (s, 3H—1a-CH_3 and 1b-CH_3); 2.82 (9, 1 H, J = 8.0 Hz); 3.98 (dd, 1H, J = 8.12 Hz/6.36 Hz, 1H).

^{13}C NMR (101 MHz, $CDCl_3$): δ (ppm) = 11.99/12.03/12.97/14.07 (2*x* 1-CH_3, 3-CH_3, and 10-CH_3)/22.64/26.85/29.22/29.46/31.82/34.40/39.13/44.23(3-C)/52.21 (1-C).

Field Tests. Experiment 1 was designed to estimate the attractiveness of synthetic potential sex pheromone components alone, or in mixtures at the ratios determined in extracts of sex pheromone glands (Table 1). Five replicates were run in both Denmark, Zeeland, in the vicinities of Roskilde, Hojby, as well as Sidinge, from July 23 to August 1, and in the vicinity of Vilnius, Lithuania, from July 29 to August 15, 1996.

On the basis of the results obtained during the first year, two subsequent experiments were conducted in 1997 near Vilnius. The first, from June 16 to July 15, was to determine the most effective ratio of 3*Z*,13*Z*-18:OH and 2*E*,13*Z*-18:Al

TABLE 1. FIELD TRAPPING OF *Sesia apiformis* MALES IN DENMARK AND LITHUANIA WITH SYNTHETIC COMPOUNDS AND THEIR MIXTURES IDENTIFIED FROM SEX PHEROMONE GLANDS OF FEMALES.

Amounts per bait (μg)			Number of males trapped ($X \pm$ SD)[a]	
3*Z*,13*Z*-18:OH	2*E*,13*Z*-18:OH	2*E*,13*Z*-18:Al	In Denmark	In Lithuania
200	2	300	3.8 ± 2.7a	3.4 ± 1.9a
200	0	300	3.4 ± 2.1a	2.4 ± 1.8a
0	2	300	0	0
200	2	0	0	0
0	0	300	0	0
200	0	0	0	0
0	2	0	0	0
0	0	0	0	0

[a] Values followed by the same letter are not significantly different; Duncan's new multiple range test, $P \leq 0.05$.

(Table 2), and the second, from June 16 to July 15, was to examine the impact of different amounts of 2*E*,13*Z*-18:OH on the effectiveness of the optimal blend of the two main components (Table 3).

Each synthetic compound tested was dissolved separately in hexane, and the required amounts were applied on red rubber caps (serum bottle cap no. 90142, Auer Bittman Soulie, Zürich) in tests run in Denmark. In Lithuania, pieces of red rubber tube, 8 mm diam, 15 mm long were used. Prior to use, dispensers were extracted in boiling ethanol $\times 3$ for 10 min and $\times 3$ for 12 hr in hexane at room temperature. Each lure was mounted in an opaque white delta trap with

TABLE 2. ATTRACTIVENESS OF 3*Z*,13*Z*-18:OH AND 2*E*,13*Z*-18:AL BLENDS FOR *Sesia apiformis* MALES IN LITHUANIA

Amounts per bait (μg)		
3*Z*,13*Z*-18:OH	2*E*,13*Z*-18:Al	Number of males trapped ($X \pm$ SD)[a]
0	300	0
30	300	5.2 ± 2.5a
90	300	7.6 ± 3.6a
200	300	8.2 ± 2.8a
300	300	5.6 ± 2.4a
300	90	0.6 ± 0.8b
300	30	0
300	0	0

[a] Values followed by the same letter are not significantly different; Duncan's new multiple range test, $P \leq 0.05$.

TABLE 3. EFFECT OF 2*E*,13*Z*-18:OH ON THE ATTRACTIVENESS OF A BINARY BLEND OF 3*Z*,13*Z*-18:OH AND 2*E*,13*Z*-18:AL IN A RATIO 2:3 TO *Sesia apiformis* MALES IN LITHUANIA

Amounts per bait (μg)			
3*Z*,13*Z*-18:OH	2*E*,13*Z*-18:OH	2*E*,13*Z*-18:Al	Number of males trapped ($X \pm$ SD)[a]
200	0	300	4.7 ± 2.3ab
200	2	300	6.1 ± 2.4a
200	20	300	3.8 ± 2.8ab
200	200	300	2.5 ± 1.6b

[a] Values followed by the same letter are not significantly different; Duncan's new multiple range test, $P \leq 0.00$.

an exchangeable bottom, coated with sticky glue. Attracon A traps with a sticky surface at the bottom (11 × 18 cm) and Pestifix glue, both from Flora Co., Tartu, Estonia, were used in Lithuania. The traps used in Denmark were smaller with a sticky surface on the bottom, 10 × 15 cm in size (Danish Institute of Plant and Soil Science, Lyngby, Denmark). Traps were fixed to tree branches in green stands of poplar, approximately 1.5 m above the ground. The distance between traps within each replicate was >10 m. Traps were inspected twice a week, and the males were counted, the sticky bottoms changed, and the traps rotated one position forward. Numbers of caught males were transformed by formula $(x + 1)^{0.5}$ and analyzed by ANOVA followed by Duncan's multiple-range test. Species identification was confirmed by genitalia analysis (Fibiger and Kristensen, 1974). Zero values of inactive lures were not included in Duncan's test. Specimens are stored in the collection of the Institute of Ecology (Vilnius, Lithuania).

RESULTS AND DISCUSSION

Analyses. Two volatile compounds, A and C, were released specifically by virgin *S. apiformis* females during their calling periods. This became obvious when GC–MS chromatograms obtained from effluvia collections by SPME during calling and noncalling periods were compared. Concentrated pentane extracts of female abdominal tips produced a gas chromatogram showing candidate compounds A, B, and C (Figure 1), which eluted under the described conditions at retention times around 18 min. Catalytic hydrogenation (Attygalle et al., 1993) of the crude extract removed the three compounds and furnished octadecanol and octadecanal, which could be easily identified by GC–MS (McLafferty and Stauffer, 1989).

Structure Elucidation of Compound A. Compound A (M = 266 by MS) showed a mass spectrum similar to that reported for 3*Z*,13*Z*-18:OH (Guo et al., 1990). Using MSTFA, the compound was silylated (Donike, 1969), indicating the

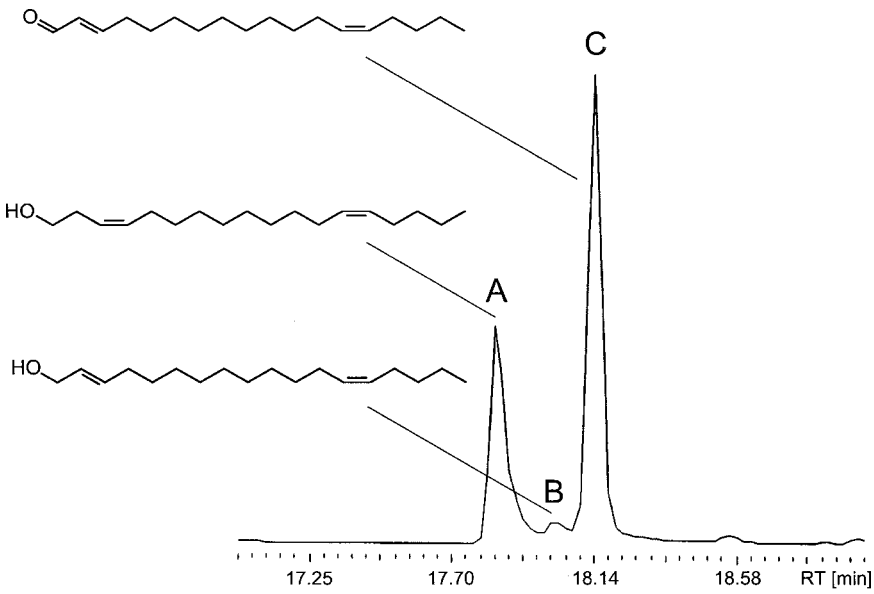

FIG. 1. Gas chromatogram of a pentane extract of abdominal tips of females of *S. apiformis* showing compounds A, B, C. See text for conditions.

presence of a hydroxy group, which suggested it to be either an octadecadienol or an octadecynol. Reaction with dimethyl disulfide produced a bis-adduct (M = 454 = 266 + 2 × MeSSMe) proving compound A to be an octadecadienol. Diagnostic ions in the mass spectrum of the bis-adduct at *m/z* 117 ($C_5H_{10}SMe$, 57%) and a complementary signal at *m/z* 289 = $M^+ - 117 -$ MeSH (29%) revealed one double bond at position 13 (Dunkelblum et al., 1985). A second pair, comprising *m/z* 105 (C_3H_5OHSMe, 28%)—the first three carbons and the attached functional groups—and its complement at *m/z* 301 = $M^+ - 105 -$ MeSH (17%) proved the second double bond at position 3. Compound A was, thus, very likely 3*Z*,13*Z*-18:OH, which had already been identified from *S. apiformis* (Kovalev et al., 1988). On GC–MS, synthetic 3*Z*,13*Z*-18:OH showed an identical retention time and the same mass spectrum as compound A. Gas chromatographic retention times of the geometrical isomers of 3*Z*,13*Z*-18:OH were different from that of compound A.

Structure Elucidation of Compound B. Compound B (M = 266 by MS) proved to be an isomer of compound A, as it could also be silylated. After reaction with dimethyl disulfide, the mass spectrum of the obtained bis-adduct (M = 454) showed some similarities to that of compound A: fragments at *m/z* 117 (56%) and *m/z* 289 (20%) established one double bond at position 13 of an octadecadienol. A signal at

m/z 91 (C_2H_3OHSMe, 21%) and a complementary one at *m/z* 315 = $M^+ - 91 -$ MeSH (5%) established the second double bond at position 2. Upon GC–MS, synthetic 2*E*, 13*Z*-18:OH showed an identical retention time and the same mass spectrum as compound B. Gas chromatographic retention times of the geometrical isomers of 2*E*, 13*Z*-18:OH were different from those of compound B.

Str*ucture Elucidation of Compound C.* Compound C (M = 264 by MS) showed a mass spectrum similar to that of 2*E*,13*Z*-18:Al (koiganal II) (Yamaoka et al., 1985). The compound could not be silylated, which excluded the presence of a hydroxy group and, therefore, it was either an octadecadienal or an octadecynal. Reaction of compound C with dimethyl disulfide afforded a mono-adduct (M = 358 = 264 + MeSSMe), the mass spectrum of which, similar to A and B, established a double bond at position 13. Analytical data strongly pointed to 2*E*,13*Z*-18:Al. Under this assumption, the formation of a single mono-adduct only during the reaction with dimethyl disulfide is explained, because electron withdrawing groups adjacent to double bonds, as in α,β-unsaturated carbonyl compounds, prohibit the addition of dimethyl disulfide (Leonhard and De Vilbiss, 1985). Nevertheless, we, tried to obtain a derivative to prove unequivocally the structure of C, and we suspected that the carbon–carbon double bond of 2-alkenals should at least react with methyl sulfide.

In a test series, commercially available 2*E*-decenal was reacted with methyl sulfide in diethyl ether. The resulting aldehyde was not only readily converted to the expected 3-methylthio derivative but also transformed into the corresponding bis(methylthio)acetal to produce 1,1,3-tris(methylthio)decane. The NMR-spectra of the compound were in accord with literature data (Rao et al., 1994 and references cited therein). The mass spectrum (Figure 2A) showed a characteristic fragmentation: a pronounced signal at M^+ = 280 (24%) accompanied by an abundant but less informative signal X at *m/z* 185 = M^+ – MeSH – MeS (17%). The highly significant parent ion Y, formed upon loss of the first two carbon units, appears at *m/z* 159 = M^+ – Z = 280 – 121. It represents the alkyl chain and the CHSMe-head keeping the charge. An important, though less abundant, diagnostic ion Z′ comprising C1 and the attached methylthio groups appears at *m/z* 107 = MeS^+ = CH – SMe (12%). Two other 2-alkenals, 2*E*-dodecenal and 2*E*-octadecenal (koiganal I, Yamaoka et al., 1985), used as additional model compounds, formed derivatives that exhibited the same fragmentation patterns and similar abundances of M^+, X, and Y. While the spectrum of 1,1,3-tris(methylthio)dodecane showed the diagnostic signal Z′ at an abundance of 13%, it was nearly absent (1%) in that of 1,1,3-tris(methylthio)octadecane (Figure 2B). Instead, the spectrum of the latter showed an intense key signal Z at *m/z* 121 (28%), which comprises C1 and C2, and represents the complementary fragment to Y (which again forms the base-peak in the spectrum). Signals at *m/z* 107 and/or *m/z* 121 may serve as diagnostic key fragments in the identification of 2-alkenals after reaction with methyl sulfide.

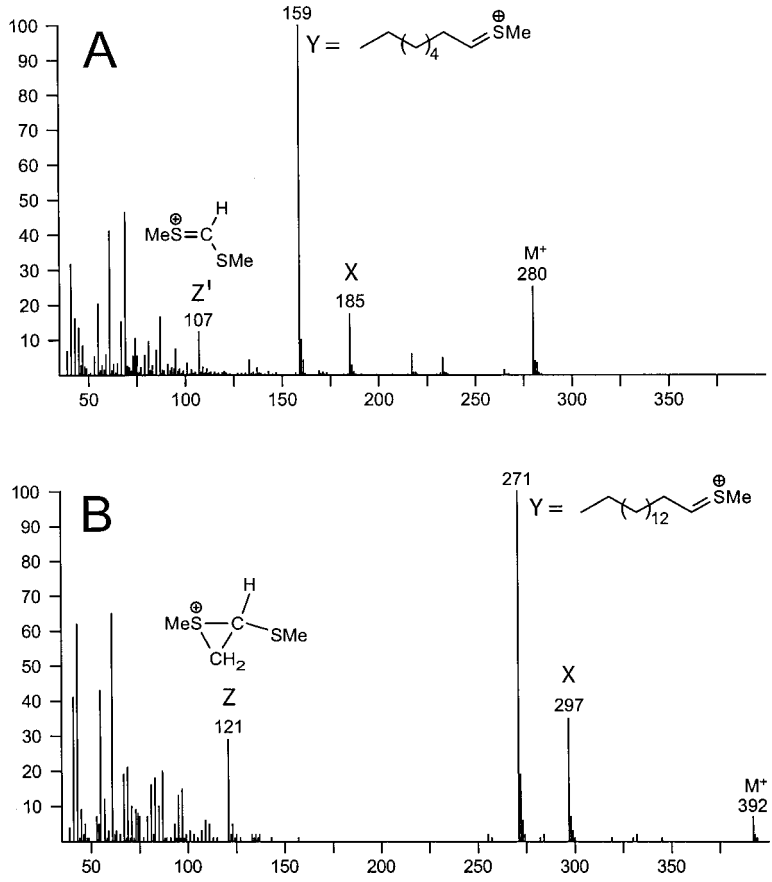

FIG. 2. Seventy electronvolt—EI mass spectra of (A) 1,1,3-tris(methylthio)decane and (B) 1,1,3-tris(thiomethyl)octadecane.

By using a solution of methyl sulfide in dimethyl sulfide under the conditions of Buser et al. (1983), 2-alkenals react almost quantitatively to form the corresponding 1,1,3-tris(thiomethyl) derivatives. This reagent may, therefore, be used advantageously in the determination of double bond positions in unsaturated compounds, as 2-alkenals are covered in addition. More detailed investigations indicate that the bis-thiomethyl acetals (the mass spectra of which are less informative) are formed prior to the addition of methyl sulfide to the double bond. Investigations with model compounds show that nonpolarized double bonds do not react with methyl sulfide under the tested conditions.

At the next step in the structure elucidation of compound C, synthetic 2*E*, 13*Z*-18:Al was reacted with a mixture of dimethyl disulfide and methyl sulfide

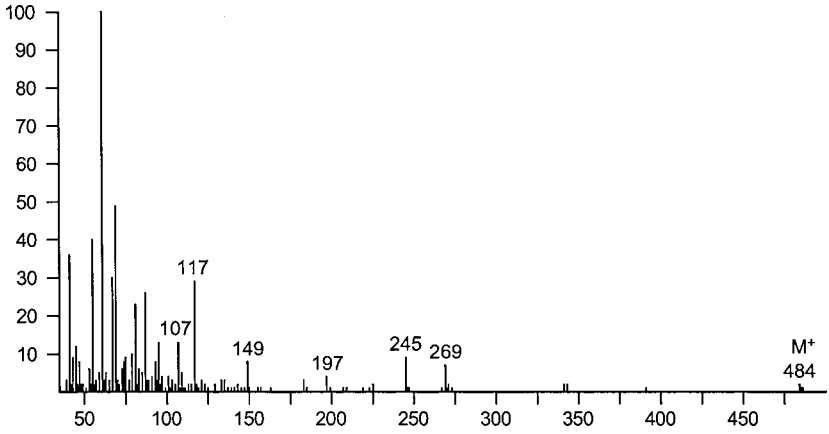

FIG. 3. Seventy electronvolt—EI mass spectrum of 1,1,3,13,14-pentakis(methylthio) octadecane. See text for interpretation of the numbered signals.

using the protocol of Buser et al. (1983). 1,1,3,13,14-Pentakis(methylthio) octadecane ($M^+ = 484$) was formed, the mass spectrum of which is shown in Figure 3. The two additional methylthio groups, introduced upon reaction of dimethyl disulfide with the double bond at C13 change the mass spectrum considerably, and fragments X and Y present in the spectra of the monounsaturated compounds are almost absent. However, the characteristic signal Z′ appears at *m*/*z* 107, while the fragment Y is hidden in *m*/*z* 269 = $M^+ - Z - MeSSMe = 484 - 121 - 94$. The additional key fragments at *m*/*z* 117 ($C_5H_{10}SMe$) and *m*/*z* 245 = $M^+ - Z - Z' - H = 484 - 121 - 117 - 1$ proved the compound to be a derivative of 2,13-octadecadienal. Signals at *m*/*z* 197 and 149 are supposed to be daughters of *m*/*z* 245, produced upon loss of one or two methyl sulfide-units, respectively, and are less informative. Reaction of the crude extract of abdominal tips of *S. apiformis* females with dimethyl sulfide/methyl sulfide yielded a product that upon GC–MS produced the same mass spectrum as the derivative of synthetic 2*E*,13*Z*-18:Al and had the same retention time. Synthetic 2*E*,13*Z*-18:Al showed the same mass spectrum and identical gas chromatographic retention time as compound C, while the corresponding data of the isomeric (3*Z*,13*Z*)-octadeca-3,13-dienal (3*Z*,13*Z*-18:Al, prepared by careful oxidation of 3*Z*,13*Z*-18:OH) was significantly different.

As mentioned in the introduction, the chemical structures and compositions of sex pheromones in Sesiidae are highly conserved: only compounds showing a Z-configured double bond at position 13—including earlier investigations on *S. apiformis* (Kovalev et al., 1988)—have been reported. The natural C18-volatiles present in our *S. apiformis* samples matched analytical data of synthetic reference

samples (as did the corresponding methylthio derivatives). Thus, we have proven that virgin females of S. *apiformis* produce 3*Z*,13*Z*-18:OH, 2*E*,13*Z*-18:OH, and 2*E*, 13*Z*-18:Al in a ratio of 200:2:300 in the sex pheromone gland. SPME collections from effluvia of calling females showed the ratio of the major compounds to be 13 ± 3%:87 ± 3% ($N = 5$), respectively, while 2*E*,13*Z*-18:OH was not detected.

Field Tests. When synthetic compounds were tested alone or as mixtures in the ratios identified in extracts of abdominal tips of females, only blends were attractive (Table 1). The traps baited with the three-component mixture, 3*Z*,13*Z*-18:OH, 2*E*,13*Z*-18:OH, 2*E*,13*Z*-18:Al in amounts of 200:2:300 μg, caught 43 males (19 of them in Denmark and 24 in Lithuania). The attractivity of the blend did not differ significantly from that of 500 μg of a 2:3 mixture of 3*Z*,13*Z*-18:OH and 2*E*,13*Z*-18:Al, which caught 34 males (17 in Denmark and 17 in Lithuania). The data obtained clearly show that 3*Z*,13*Z*-18:OH and 2*E*,13*Z*-18:Al are essential sex pheromone components of *S. apiformis*.

Field screening tests of blends of 3*Z*,13*Z*-18:OH and 2*E*,13*Z*-18:Al in different ratios showed that *S. apiformis* males were strongly attracted to the blends containing alcohol and aldehyde in amounts from 30:300 to 300:300 μg (Table 2). Numbers of insects trapped with these blends did not differ significantly. The range of ratios that proved effective in the field trials covered both the 2:3-proportion found in pentane extracts and the average 1:7-release rate determined from calling females.

Addition of 2 or 20 μg of 2*E*,13*Z*-18:OH to 500 μg of the 2:3 mixture of 3*Z*,13*Z*-18:OH and 2*E*,13*Z*-18:Al had no significant effect on the attraction of males (Table 3). However, when 200 μg of 2*E*,13*Z*-18:OH were added to 500 μg of the binary mixture, the attractiveness of the lure was significantly reduced. The biological significance of 2*E*,13*Z*-18:OH in *S. apiformis* remains unknown. It may have a function as an interspecific signal. It certainly is biosynthetically related to 2*E*,13*Z*-18:Al.

In *S. apiformis*, an aldehyde was identified for the first time as a component of the sex pheromone in the Sesiidae. Previously, synthetic 3*Z*,13*Z*-18:Al, an isomer of 2*E*,13*Z*-18:Al, was reported to elicit high EAG response in one *Podosesia* and two *Synanthedon* species (Nielsen et al., 1979), but it is unclear whether the unstable 3*Z*,13*Z*-18:Al would at least in part rearrange under field conditions, causing presence of the more stable 2*E*,13*Z*-18:Al in the tested samples. Field screenings in Hungary, using 2,13- and 3,13-octadecadienal alone and in binary mixtures with corresponding alcohols or acetates, did not reveal any activity of aldehydes as attractants or attraction synergists for sesiids (Szöcs et al., 1989). However, aldehydes may well be present in pheromone systems of Sesiidae other than *S. apiformis.*

To date, 2*E*,13*Z*-18:Al had only been identified as a pheromone compound in the clothes moth *Tineola bisselliella* (Hum.) (Tineidae) (Yamaoka et al., 1985;

Takacs et al., 2001). The identification of 3*Z*,13*Z*-18:OH and 2*E*,13*Z*-18:Al in *S. apiformis* confirms the coincidence of chemical structures as sex pheromone components within Sesiidae and Tineidae, which was noted earlier (Szöcs et al., 1989; Būda et al., 1993). For monitoring the species, we suggest using a blend of 3*Z*,13*Z*-18:OH and 2*E*,13*Z*-18:Al in a ratio of 2:3 and at a dose range of 100–500 μg, applied on rubber septa in sticky traps.

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IDENTIFICATION OF TWO SEX PHEROMONE COMPONENTS OF THE POTATO APHID, *Macrosiphum euphorbiae* (THOMAS)

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Abstract—Females of the potato aphid *Macrosiphum euphorbiae* exhibit typical calling behavior, with virgin female oviparae raising their back legs off the substrate to release sex pheromone from glands on the tibia. Airborne collections from calling oviparae were analyzed by GC and GC-MS to determine if, like the majority of aphids examined to date, they produced (1*R*,4*aS*,7*S*,7*aR*)-nepetalactol (**1**) and (4*aS*,7*S*,7*aR*)-nepetalactone (**2**). Both components were present and produced in ratios that varied with age from 4:1 to 2:1. The relative stereochemical configurations of these components were determined by GC-coinjection of the aphid-derived sample with synthetic standards on both HP-1 and DB-Wax GC columns. The absolute stereochemical configuration of the nepetalactol (determined from approximately 15 μg of material in an air entrainment sample) was determined as (1*R*,4*aS*,7*S*,7*aR*)-**1** by derivatization of the aphid sample with (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (Mosher's acid chloride) to generate a diastereoisomer that was compared to synthetic samples by NMR spectroscopy and GC. In bioassays in the wind tunnel, *M. euphorbiae* males responded to potato plants with oviparae but not to unattacked plants or those infested with parthenogenetically reproducing apterae. In no-choice laboratory bioassays, the same level of male response was observed to virgins and to the 3:1–5:1 synthetic blends of nepetalactol (**1**):nepetalactone (**2**). However, the time taken to reach the source was significantly less

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to virgin females than to the synthetic pheromone blends. In all cases, males walked rather than flew to the source. Males showed lower responses to a 1:1 synthetic mixture and did not respond to either of the components when presented alone. Under field conditions, few *M. euphorbiae* males were captured in traps baited with different ratios of the synthetic pheromone. Possible reasons for the different responses under laboratory and field conditions are discussed.

Key Words—Potato aphid, *Macrosiphum euphorbiae*, sex pheromone, wind tunnel, (1*R*,4*aS*,7*S*,7*aR*)-nepetalactol, (*4aS*,7*S*,7*aR*)-nepetalactone, calling female.

INTRODUCTION

Many aphid species (Homoptera: Aphididae) have complex seasonal reproductive cycles, with a number of parthenogenetically reproducing asexual generations during the summer and one autumn generation of sexually reproducing morphs that produces the overwintering eggs (Moran, 1992). They may be monoecious, exploiting the same host throughout the year, or heteroecious, alternating between primary overwintering and secondary summer hosts. The asexual summer generations of aphids are among the most serious pests of agricultural and horticultural crops in the world, not only from the direct effects of feeding but also as vectors of plant diseases (Kennedy et al., 1962; Blackman and Eastop, 2000; Radcliffe and Ragsdale, 2002). Current aphid control is strongly dependent on the use of insecticides, but because this carries the undesired side effects of insecticide resistance, reduction of beneficial insect populations, and potential environmental contamination (Metcalf, 1980; Hemingway et al., 2002; Foster et al., 2003), alternative approaches must be sought.

Sexual female aphids can attract males with a sex pheromone released from the hind tibiae (Pettersson, 1970, 1971; Marsh, 1972; Eisenbach and Mittler, 1980; Dawson et al., 1990; Lilley and Hardie, 1996; Boo et al., 2000), and the pheromones of many aphid species in the subfamily Aphidinae have been identified (Dawson et al., 1987, 1988, 1989; Campbell et al., 1990; Lilley et al., 1994/1995; Gabrys et al., 1997; Boo et al., 2000). Whereas aphid sex pheromones have several potential uses, such as monitoring aphid populations, manipulating aphid parasitoids, or disseminating pathogens (Campbell et al., 1990; Hardie et al., 1992; Lilley et al., 1994; Pickett et al., 1994; Gabrys et al., 1997; Hartfield et al., 2001), they are as yet unexploited. This is due in part to the fact that the sexual morphs occur late in the season rather than in the summer when crops are being invaded by the asexual alate females. However, if sex pheromones are to be used effectively, then understanding the pheromone biology for the species in question is essential (see McNeil, 1991, and references therein).

The potato aphid, *Macrosiphum euphorbiae* (Thomas), can be an important pest of potatoes and tomatoes (Shands et al., 1965; Lange and Bronson, 1981), damaging plants by sucking phloem sap and by transmitting viral diseases

(Kennedy et al., 1962; Alyokhin et al., 2002). In this paper, we undertook studies to determine whether *M. euphorbia* produces (1*R*,*4aS*,7*S*,7*aR*)-nepetalactol (**1**) and (*4aS*,7*S*,7*aR*)-nepetalactone (**2**), the two sex pheromone components found in the majority of aphids studied to date. We also examined the rates of emission as a function of female age, and tested male responses to putative female sex pheromone blends under controlled conditions in a wind tunnel and in the field.

METHODS AND MATERIALS

Insects. Aphids used in these experiments came from an annually restocked, multiclonal laboratory culture that was established from parthenogenetic individuals collected from potato fields near Quebec City. Colonies were maintained on potato seedlings, *Solanum tuberosum* c.v. Norland, at $20 \pm 1^{\circ}$C, $60 \pm 10\%$ RH, under a 16L:8D photoperiod, conditions that ensure continuous asexual production. Potato seedlings at the 4–6 leaves stage were used as host plants in all experiments, and new plants were provided every 3 d. Sexuals were obtained by rearing two consecutive generations at $20 \pm 1^{\circ}$C, $60 \pm 10\%$ RH under a 10L:14D photoperiodic regime. Gynoparous (which produce the sexual females) and androparous (which produce the winged males) females 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. The sexes were maintained separately to ensure that females were virgin and that males had never been exposed to pheromone prior to being used in bioassays.

Experimental Chemistry. ^{1}H, ^{13}C, and ^{19}F NMR spectroscopy was performed using a Bruker 500 Avance NMR spectrometer with ^{1}H referenced to $CDCl_3$ (7.25 ppm), ^{13}C to $CDCl_3$ (77.0 ppm), and ^{19}F to $CFCl_3$ (0 ppm). Quantitative ^{1}H NMR spectroscopy was performed using a pulse angle of 30°, an acquisition time 5T1 (with T1 measured to be 2.5 sec) and a delay of 5 sec.

Isomers of nepetalactol derived from the essential oil of *Nepeta cataria* or from synthetic preparation (20 mg, 0.12 mmol) were dissolved in dichloromethane (1 ml) under nitrogen. A solution of (*S*)-(+)-α-methoxy-α-(trifluoromethyl) phenylacetyl chloride (40 mg, 0.16 mmol) and pyridine (25 μl) in dichloromethane (1 ml) prepared under nitrogen was added, together with a few crystals of dimethylaminopyridine, and the reaction was stirred overnight. NMR analysis of an aliquot showed complete esterification of the lactol. The solvent was removed under a stream of nitrogen and the residue purified on a florisil column eluted with 10% diethyl ether in petroleum ether (40–60°C boiling fraction). Fractions containing the lactol ester were combined and evaporated to dryness.

(*1S,4aS,7S,7aR*)-*Nepetalactolyl* (*S*)-(+)-*α-methoxy-α-trifluoromethylphenylacetate (**3**)*: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.52 (2H, m, *ortho*-H aromatic), 7.40–7.37 (3H, m, *meta*- and *para*-H aromatic), 6.20 (1H, d, $J = 2.9$ Hz, H-1), 5.87 (1H, br s, H-3), 3.52 (3H, s, OCH_3), 2.46 (1H, br q, $J = 6.2$ Hz, H-4a), 1.90

(1H, m, H-5), 1.87 (1H, m, H-7), 1.84 (1H, m, H-6), 1.79 (1H, m, H-7), 1.51 (1H, m, H-5), 1.42 (3H, s, 4-Me), 1.19 (1H, m, H-6), 1.08 (3H, d, $J = 6.4$ Hz, 7-ME); ^{13}C NMR (125 MHz, $CDCl_3$) δ 165.6 (CO), 133.1 (C3), 131.8 (C1″), 129.6 (C4″), 128.3 (C3″), 127.6 (C2″, q, $J = 1.9$ Hz), 123.2 (C3′, q, $J = 286.6$ Hz), 114.3 (C4), 93.3 (C1), 84.6 (C-2, q, $J = 27.6$ Hz), 55.3 (OCH_3), 48.9 (C7a), 36.7 (C4a), 34.9 (C7), 33.0 (C6), 28.7 (C5), 19.6 (7-Me), 15.7 (4-Me); ^{19}F NMR (470 MHz, $CDCl_3$) $\delta -72.69$ (CF_3).

(*1R, 4aR, 7R, 7aS*)-*Nepetalactolyl* (*S*)-(+)-*α-methoxy-α-trifluoromethylphenylacetate* (**4**): ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.55 (2H, m, *ortho*-H aromatic), 7.40–7.35 (3H, m, *meta*- and *para*-H aromatic), 6.16 (1H, d, $J = 3.5$ Hz, H-1), 5.98 (1H, br s, H-3), 3.56 (3H, s, OCH_3), 2.44 (1H, br q, $J = 6.7$ Hz, H-4a), 1.89 (1H, m, H-5), 1.85 (1H, m, H-7), 1.84 (1H, m, H-6), 1.73 (1H, m, H-7a), 1.50 (3H, s, 4-Me), 1.49 (1H, m, H-5), 1.17 (1H, m, H-6), 1.00 (3H, d, $J = 6.2$ Hz, 7-Me); ^{13}C NMR (125 MHz, $CDCl_3$) δ 165.7 (CO), 133.3 (C3), 132.1 (C1″), 129.6 (C4″), 128.3 (C3″), 127.4 (C2), 123.2 (C3′, q, $J = 288.6$ Hz), 114.6 (C4), 93.4 (C1), 84.3 (CF_3, q, $J = 27.8$ Hz), 55.4 (OCH_3), 48.5 (C7a), 37.0 (C4a), 34.8 (C7), 33.0 (C6), 28.9 (C5), 19.6 (7-Me), 15.7 (4-Me); ^{19}F NMR (470 MHz, $CDCl_3$) $\delta -72.14$ (CF_3).

Purified samples of **3** and **4** were analyzed on a Hewlett-Packard 5880 GC, fitted with a nonpolar HP-1 capillary column (40 m, 0.32 mm ID, 0.52-μm film thickness), a cool-on-column injector, and a flame ionization detector (FID). The GC oven temperature was maintained at 40°C for 1 min and then raised by 10°C/min to 200°C. The carrier gas was nitrogen. The retention time for **3** was 33.40 and **4** was 32.50, for an α value of 1.028.

Derivatization of Volatiles Sample from M. euphorbiae. Air entrainment samples of *M. euphorbiae* oviparae (see below), containing 15 μg of nepetalactol by GC approximation, were combined, concentrated under a stream of nitrogen, and dissolved in dichloromethane (0.5 ml) under nitrogen. A solution of (*S*)-(+)-*α*-methoxy-*α*-(trifluoromethyl)phenylacetyl chloride (40 mg, 0.16 mmol) and pyridine (25 μl) in dichloromethane (0.5 ml) prepared under nitrogen was added, together with a few crystals of dimethylaminopyridine, and the reaction was stirred overnight. The solvent was removed under a stream of nitrogen and the residue partially redissolved in 10% diethyl ether in petroleum ether (40–60°C boiling fraction). The insoluble material was discarded and the soluble portion decanted. The solvent was removed under a stream of nitrogen and the residue redissolved in $CDCl_3$ for NMR and GC analysis.

Isolation of Volatiles. Twenty five virgin oviparae of *M. euphorbiae* (1-d old) were placed on a potato seedling in a glass culture vessel (Quickfit FV range, 1 l). Air that had been dried and purified by passage through an activated 5 Å molecular sieve and charcoal, was drawn at 1 l/min through the container. Volatiles were trapped on Porapak Q that had been cleaned by washing with ether (5 ml) and heating at 150°C for 12 hr under a stream of nitrogen. Because preliminary

tests showed that the quantities of pheromone released were very low, subsequent collection was carried out over a 4-d period. The volatiles were eluted from the Porapak Q with freshly distilled ether (700 μl). The resulting extract was stored in a sealed glass ampoule at -20°C. The procedure was repeated for two additional 4-d periods, replacing the host plant each time, to test for possible age-related changes in pheromone emission. Females may live up to 4 wk, but we limited sampling to the first 12 d as mating occurs within 48 hr under laboratory conditions and it seemed unlikely that a female would remain unmated for any greater length of time in nature. There were two sets of controls, with volatiles being collected from an uninfested potato plant, and one infested with 25 apterous viviparae. The entire experiment was replicated three times.

Analysis of Volatiles. Air entrainment samples were analyzed by GC on both polar (DB-wax, 30 m $\times$ 0.32 mm ID $\times$ 0.5 μm film thickness) and nonpolar (HP-1, 50 m $\times$ 0.32 mm ID $\times$ 0.5 μm film thickness) capillary columns using a HP5890 GC (Agilent Technologies, UK) fitted with a cool-on-column injector and FID. The GC oven temperature for both columns was maintained at 40°C for 1 min after sample injection and then raised by 10°C/min to 240°C. The carrier gas was hydrogen. Further analysis by GC-MS was performed on a VG Autospec instrument (VG Analytical, UK) coupled to a HP5890 GC fitted with an HP-1 column.

Wind Tunnel Bioassays. Experiments were conducted in a laminar airflow wind tunnel (290 cm long, 120 cm wide, and 100 cm high) located in an environmental chamber maintained at 18 $\pm$ 0.5°C with RH 40–50%. Tunnel lighting was provided by eight (40 W) fluorescent light bulbs uniformly placed above the tunnel ceiling, giving a light intensity of 150 lux where bioassays were carried out. All assays, using 5- to 6-d-old naïve, virgin males, were carried out in the third to sixth hours of the photophase, as this was the period of maximum female calling activity under controlled laboratory conditions (Goldansaz and McNeil, 2003). Males were considered unresponsive if they did not respond within 3 min. Individuals were used only once. However, under these conditions whenever males took flight they flew downwind rather than upwind. Therefore, we connected the platforms bearing the source and the release cage with a thin string bridge, a technique previously used by Eisenbach and Mittler (1980), and repeated the experiment described above. This "bridge" approach was also used to test the response of *M. euphorbiae* males to artificial sex pheromone. Six ratios of the two compounds were tested: 1:0, 0:1, 1:1, 3:1, 4:1, and 5:1, together with the ether solvent as a control, using a randomized block design with three replicates of 15 males. Our standard solutions were 1 mg/ml so, for example, to obtain the 1:1 ratio we applied 1 μl of each solution (1 μg of each compound) onto a 1 cm diam filter paper disc. These preparations were replaced every 15 min.

Testing Synthetic Pheromone Lures Under Field Conditions. A series of 08-CPV Chromacol glass vials were loaded with 10 mg of either (1*R*,4*aS*,7*S*,7*aR*)-nepetalactol (**1**) or (4*aS*,7*S*,7*aR*)-nepetalactone (**2**) in 50 μl Et_2O. A 1-mm hole

was drilled in the top which provided a 200 μg per day release rate (Gabrys et al., 1997). A series of water traps made from clear plastic petri dishes were baited with 1:0, 0:1, 1:1, and 5:1 nepetalactol:nepetalactone lures, whereas vials with solvent only were used in the controls. Thus, in the 5:1 treatment, there were 5 vials of nepetalactol and 1 of nepetalactone, whereas the control had 6 with Et_2O. In this case, the actual release rates (**1**:**2**) would be approximately 1.0:0.2 mg/d. The traps were placed along the edges of commercial potato fields near Pont Rouge, QC, and near a rose garden in the Jardin Van Den Hende on the Laval University Campus in 1997, 1998, and 2000. There were a minimum of three sites each year, with five traps per site. The traps, positioned 1 m above ground on wooden stakes 10 m apart, were checked at least every 3 d from July through October. At each inspection, all material was collected and the traps were refilled with soapy water. Although the pheromone blends have a constant release rate over 5 wk at 20°C (Gabrys et al., 1997), to ensure consistency under field conditions the lures were changed every 2 wk.

Statistical Analyses. Differences in the ratio of the two pheromone components as a function of female age were compared using a permutation test (Legendre and Legendre, 1998). Categorical responses (orient/not orient; take flight/not taking flight; reach the source/not reach the source) of males exposed to different odor sources were compared with a logit model using the GENMOD procedure of SAS, whereas the time to reach the source was compared with ANOVA using GLM procedure (SAS, 1999). The proportion of males orienting in the presence or absence of a bridge was compared with an analysis of contrast, using the GENMOD procedure (SAS, 1999). The latency times for males to respond to various odor sources were compared by contrast analysis, using the GLM procedure (SAS, 1999).

RESULTS

Pheromone Identification. GC analysis of the air entrainment samples from virgin sexual females of *M. euphorbiae* showed the presence of two peaks that were identified by coupled GC-MS and GC coinjection, on two achiral columns (HP-1 and DB-WAX), as identical to nepetalactol and nepetalactone derived from *N. cataria* essential oil. These plant-derived compounds were used for the field and laboratory studies, with nepetalactone isolated from the essential oil of *N. cataria*, and nepetalactol obtained from borohydride reduction of nepetalactone. The relative stereochemical configuration has been defined previously by chemical synthesis and NMR spectroscopy (Dawson et al., 1996; Hooper et al., 2002). The enantiomers of these compounds, however, could not be separated by GC on a chiral stationary phase (β-cyclodextrin). To demonstrate enantiomeric purity, synthetically produced enantiomers (1*R*,4*aS*,7*S*,7*aR*)-nepetalactol and (1*S*,4*aR*, 7*R*,7*aS*)-nepetalactol were esterified separately with (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetylchloride to generate diastereoisomers (**3**) and (**4**),

respectively, as synthetic standards (Figure 1). After derivatization of nepetalactol from *N. cataria*, NMR analysis showed the plant product to be (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**1**) with an enantiomeric excess (e.e.) of >99% by quantitative ^{1}**H** NMR spectroscopy. The e.e. measured before and after purification was the same.

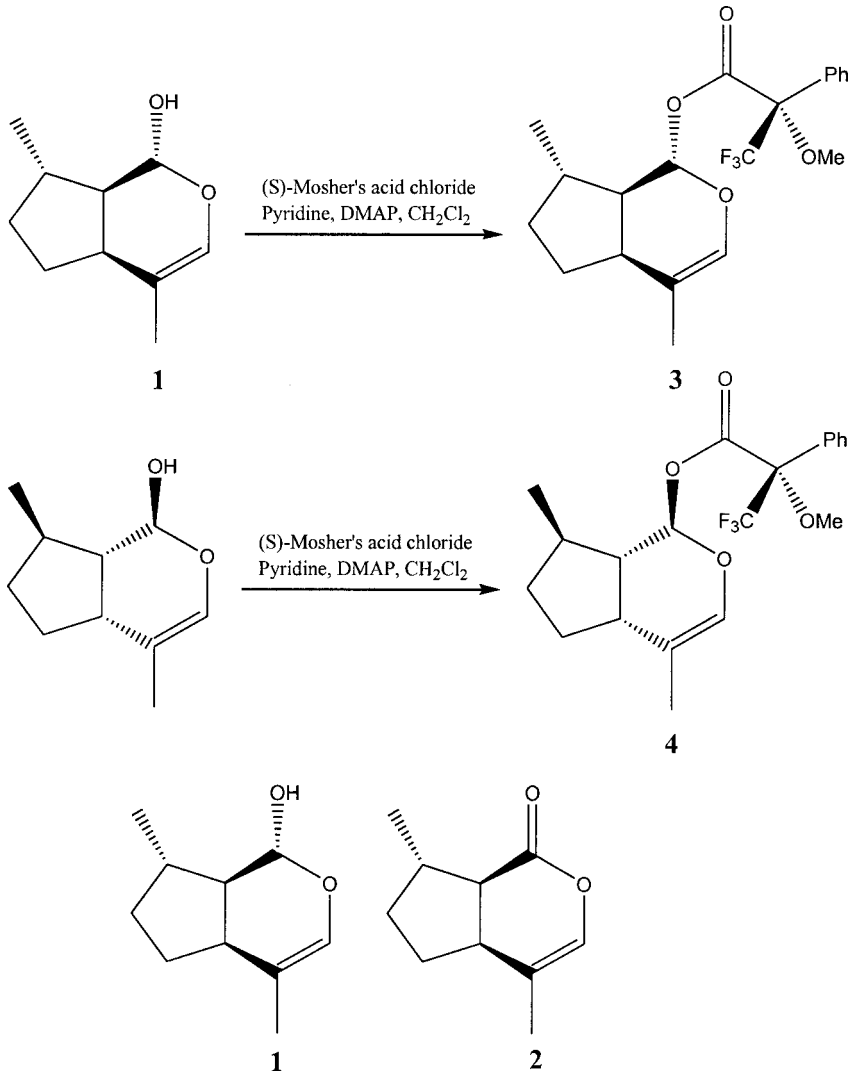

FIG. 1. Transformation of (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol and its enantiomer to the corresponding Mosher's esters.

There are errors in NMR integrative determination of enantiomeric excess, indeed the commercial (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride is marketed with an e.e. of 98%, but the experiment clearly shows the plant-derived nepetalactol possesses very high or complete enantiospecificity.

The air entrainment sample of *M. euphorbiae* contained many components, and the total sample before derivatization contained approximately 15 μg of nepetalactol. An overnight accumulation by ^{1}**H** NMR spectroscopy after derivatization of the sample revealed H-1 and H-3 proton resonances of the desired compound, which could be observed in a part of the spectrum clear of other resonances (Figure 2). By comparison with synthetic standards **3** and **4** (Figure 2), it was clear that *M. euphorbiae* produced predominantly (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol. The absolute stereochemical configuration of nepetalactone from *M. euphorbiae* is presumed to be the same because the inversion of three chiral centers would not be expected. As added proof, GC-coinjection of the synthetic standards **3** and **4** with the derivatized air entrainment sample was performed on an HP-1 column. The trace from the derivatized *M. euphorbiae* sample contained a peak that coeluted with **3** and no peak in the trace coeluted with **4**.

The ratio of **1** and **2** from *M. euphorbiae* appeared to change as a function of age, averaging *ca.* 4:1 on days 1–4, *ca.* 3:1 and 2:1 on days 5–8 and 9–12, respectively. However, these differences were not statistically significant ($P = 0.18$). Assuming that all females were calling, and taking into account the duration of the calling period (see Goldansaz and McNeil, 2003), then the hourly nepetalactol:nepetalactone release rates ranged from 50:15 to 20:10 ng/aphid/hr over the 12-d collection period. Insufficient material was obtained from the entrainments to conduct bioassays.

Bioassays. In the absence of any odor source, and with no bridge for individuals to walk upwind, males moved to the top of the release cage and flew downwind. In contrast, when any odor source was present, males that exhibited orientation behavior moved to the upwind edge of the release cage to face the source, walked down the side, and moved back and forth on the upwind edge of the platform. However, as before, when they took flight, all males flew downwind. There was a shorter latency period before taking flight in clean air than when an odor source was present ($F = 18.57$, df = 1, 6, $P = 0.005$; Table 1). *M. euphorbiae* males oriented upwind more when exposed to calling virgin females than to asexual females or a potato plant ($\chi^2 = 83.20$, df = 3, $P < 0.001$; Figure 3). Furthermore, males exposed to calling oviparae spent longer orienting than to other odor sources ($F = 18.22$, df = 1, 6, $P = 0.005$; Table 1), resulting in fewer individuals actually taking off during the assay ($\chi^2 = 40.12$, df = 3, $P < 0.001$; Figure 3a). When a bridge was available between the source and release platforms, the percentage of males orienting to the different sources was similar to those seen in the previous experiment ($\chi^2 = 0.23$, df = 1, $P = 0.64$), and for any given

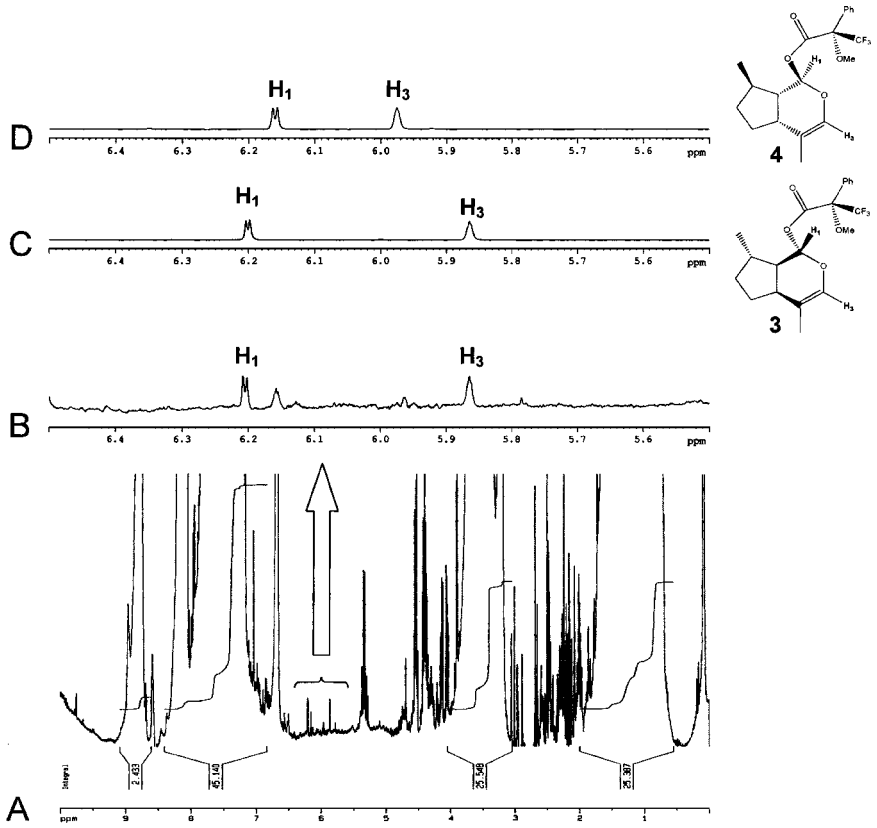

FIG. 2. (A) ^{1}**H** NMR spectrum of the derivatized air entrainment sample from *M. euphorbiae* showing (B) H-1 and H-3 resonances for the derivatized nepetalactol. Comparison with synthetic standards (C and D) shows the aphid-produced compound is identical to (1*R*,4*aS*,7*S*,7*aR*)-nepetalactol (**1**).

odor source the latency times did not differ significantly ($F = 2.20$, df $= 1, 12$, $P = 0.16$; Table 1). In this case, more males walked to calling oviparae on plants than parthenogenetic females or plants only ($\chi^2 = 70.75$, df $= 3$, $P < 0.001$; Figure 3b). However, even when males had the possibility to walk to the source, there was some variability in response to calling females, because some individuals still took flight and subsequently moved downwind (Figure 4). The percentage of males orienting to and reaching the source when exposed to 3:1, 4:1, and 5:1 nepetalactol (**1**):nepetalactone (**2**) lures was similar to those observed when virgin females were used (Table 2). The responses were significantly lower when a 1:1

TABLE 1. DURATION ($\bar{x} \pm$ SE) OF THE LATENCY PERIOD (SEC) PRIOR TO TAKING FLIGHT OR WALKING UPWIND IN *Macrosiphum euphorbiae* MALES WHEN EXPOSED TO DIFFERENT ODOR SOURCES IN A LABORATORY BIOASSAY

Source	Without a bridge	With a bridge
Clean air	67.9 ± 7.6	67.5 ± 5.4
Uninfested potato plant	84.4 ± 2.7	94.4 ± 5.4
Plant with virginoparae	86.2 ± 4.9	89.7 ± 1.4
Plant with oviparae	100.9 ± 11.9	116.1 ± 15.2

blend was used and little response was observed when either of the components was presented alone. In all cases, the time taken to reach the synthetic lures was greater than to virgin oviparae (Table 2).

Field Trapping. We captured in excess of 2000 male aphids, with representatives of all the major genera of aphids found in Quebec. However, most could

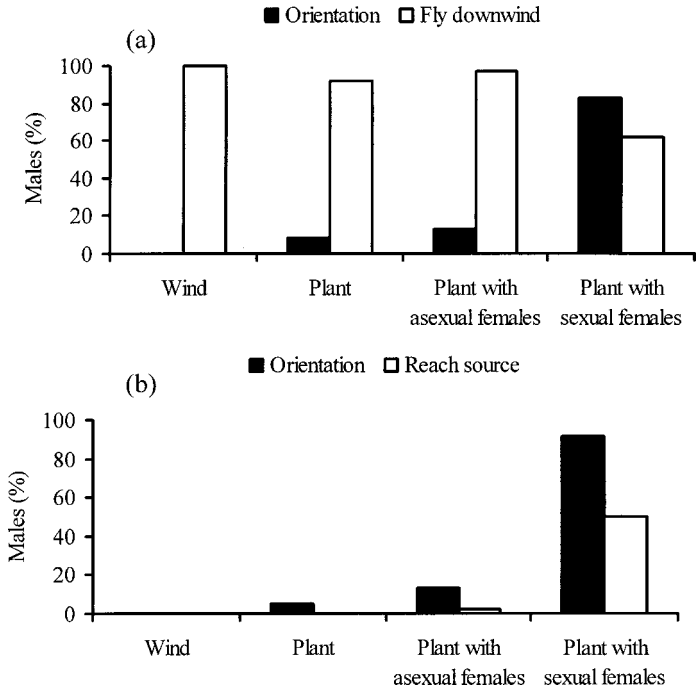

FIG. 3. Percent of *M. euphorbiae* males exhibiting orientation and flight response to different odor sources in a wind tunnel (wind speed 40 cm/sec) when there was (a) no physical connection and (b) a physical connection between the release site and the source.

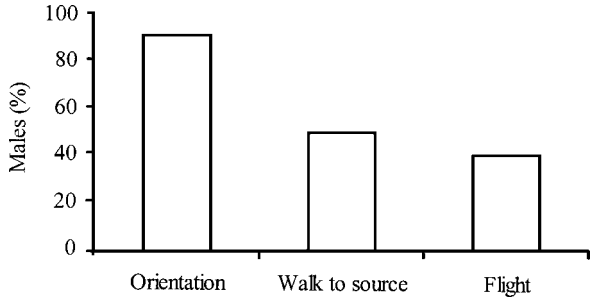

FIG. 4. Percent of *M. euphorbiae* males orienting, walking to the source, or flying downwind when exposed to five calling oviparae in a wind tunnel (wind speed 40 cm/sec) when there was a physical connection between the release site and the source.

not be identified with any certainty beyond the family or genus, because males for most species have not been previously described (Eastop, personal communication). Only three were *M. euphorbiae*, and all were captured near potato fields in late August, in traps baited with a 5:1 nepetalactol:nepetalactone lure, which of all the combinations used was closest to the ratio emitted by young virgin females.

TABLE 2. BEHAVIORAL RESPONSES ($\bar{x} \pm$ SE) OF *Macrosiphum euphorbiae* MALES WHEN EXPOSED TO CALLING FEMALES AND SYNTHETIC PHEROMONE LURES, DIFFERING IN THE RATIO OF NEPETALACTOL: NEPETALACTONE, UNDER CONTROLLED CONDITIONS IN A WIND TUNNEL

Ratio (I:II)	Orienting to source (%)[a]	Reaching source (%)[a]	Time to reach source (sec)[a]
0:0	0 a	0 a	—
0:1	6.0 ± 2.8 ab	0 a	—
1:0	14.0 ± 2.8 b	0 a	—
1:1	47.0 ± 12.3 c	24.6 ± 8.5 b	181.2 ± 9.8 a
3:1	94.0 ± 2.7 e	53.0 ± 7.5 c	174.6 ± 6.6 a
4:1	92.0 ± 0.5 de	53.3 ± 3.4 c	187.5 ± 3.1 a
5:1	75.0 ± 0.0 d	44.3 ± 5.8 c	193.7 ± 0.9 a
Oviparae	80.0 ± 4.6 de	50.0 ± 4.7 c	114.9 ± 9.6 b

[a] Values within a column followed by different letters are significantly different ($P < 0.05$).

DISCUSSION

Macrosiphium euphorbiae oviparae produce both (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**1**) and (4a*S*,7*S*,7a*R*)-nepetalactone (**2**), the same two major components reported for a number of other aphid species. The absolute stereochemistry of **1** has been elucidated from this complex mixture of aphid and host plant volatiles using microchemistry and NMR spectroscopy on only 15 μg of material. It is unusual to use NMR to determine the structure of components in complex mixtures and more so at this microscale. The unusual chemical shift of the derivative allowed NMR analysis in a clear window of the NMR spectrum (Figure 1), and this method can probably be extended to the analysis of other aphid-produced nepetalactols. It has been proposed that different ratios of the two compounds, ranging from 1:29 in *Aphis fabae* (Pickett et al., 1992) to 30:1 in *Cryptomyzus* spp. (Guldemond et al., 1993), may provide species-specific pheromones (Guldemond et al., 1993; Hardie et al., 1994; Thieme and Dixon, 1996). The results of the present study lend support to this hypothesis, as *M. euphorbia* oviparae release a different ratio from those reported for other species studied to date. Furthermore, in laboratory assays males responded to 3:1–5:1 ratios of the components in synthetic lures (similar to the variability seen in the pheromone ratio from different aged virgin females) but significantly less to the other ratios, whereas each component alone was virtually inactive.

However, the 5:1 ratio which proved attractive in the laboratory was not effective in attracting males during 3 years of field trials. There are nonexclusive explanations to account for the marked differences between laboratory and field results. The first relates to population size. The numbers of male *M. euphorbiae* captured in suction traps each fall in a previous study carried out over several years at different locations were always low (<10/year) in Quebec (Conrad Cloutier, personal communication). This was markedly different from the high densities of males from other aphid species of agricultural importance, suggesting that densities of *M. euphorbiae* sexuals seldom reach high densities in the study area.

A second explanation relates to the quality and/or quantity of pheromone actually released, which may vary considerably between controlled laboratory and variable field conditions. Pheromone stereochemistry may significantly influence male aphid responses (see Hardie et al., 1997) although in the present study on *M. euphorbia* the enantiomeric purity of the nepetalactone and nepetalactol of the lures and from calling females were predominantly the same. However, because of the extremely small quantity of aphid-produced material available, the NMR analysis (Figure 2) could not rule out the possibility that minor stereoisomers of nepetalactol were present but not detectable at this level of sensitivity. Furthermore, there is the possibility of presently unknown minor components. For example, nepetalactone is the only compound presently identified from the oviparae of *Brevicoryne brassicae* (Gabrys et al., 1997), *Sitobion avenae* (Lilley et al., 1994/1995), and *S. fragariae*

(Hardie et al., 1992), and alone would not provide a species-specific signal if all species occurred simultaneously in the same general habitat. Also, synthetic sex pheromones, in the ratios reported from calling females, were unattractive to males of *Aphis fabae* and *Acyrthosiphon pisum* (Hardie et al., 1990), as well as to *Cryptomyzus galeopsidis* males (Thieme and Dixon, 1996). The fact that *M. euphorbiae* males took significantly less time to reach calling females than any of the synthetic lures in the laboratory assays suggests some aspect of the pheromone blend may remain to be elucidated.

A third explanation may relate to the manner in which males actually reach a pheromone source. As seen in the bioassays (Figure 3), under a constant wind speed of 40 cm/sec males did not fly upwind to a pheromone source. Furthermore, studies examining the effect of wind speed under laboratory and field conditions (Goldansaz and McNeil, unpublished) showed that even at low velocities, *M. euphorbiae* males often walked rather than flew upwind to calling females. If walking behavior is a major component of mate location in potato aphid males, then they would be unable to follow a plume upwind by walking because the traps were supported on stakes 1 m above ground. Campbell et al. (1990) captured hop aphid, *P. humuli*, males 20 m from the hop garden, indicating males move greater distances than the few centimetres initially proposed (see Dawson et al., 1990). However, it is not clear what proportion of such movement is due to passive transport on air currents compared with active oriented flight to the pheromone source. Logically, one would expect volatiles from a suitable primary host plant to be more effective at long distance than the sex pheromone emitted by a small calling female, so host plant volatiles, alone or in concert with the female sex pheromone, could provide males more specific long distance cues for mate location. Increased trap catches have been reported for the hop aphid, *Phorodon humuli*, and bird-cherry aphid, *R. padi* (Campbell et al., 1990; Hardie et al., 1994), when host plant volatiles were combined with the pheromone. However, this is not always the case with host plant volatiles acting as low ranking cues for males of *Cryptomyzus* spp. and two subspecies of *A. fabae* (Guldemond et al., 1992; Thieme and Dixon, 1996). Although *M. euphorbiae* males showed positive bioassay responses in the absence of primary host plants under laboratory conditions, one cannot rule out that host plant volatiles from the primary host may be an important cue for males searching for mates under field conditions.

To date, we do not have a sufficiently solid understanding of the pheromone ecology of *M. euphorbiae*, or of any other aphid species, to effectively exploit female sex pheromones in aphid management programs. Future research comparing different species should include investigations of the chemical and ecological aspects of female pheromone production and release, as well as the male behaviors when locating pheromone sources. In addition, the importance of potential male pheromones, auditory signals, and physical factors such as size, shape, color, including species-specific courtship behaviors (Hardie et al., 1990; Steffan, 1990)

that may influence mate choice once the two sexes are in close proximity, need to be clarified.

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TRILINOLEIN IDENTIFIED AS A SEX-SPECIFIC COMPONENT OF TERGAL GLANDS IN ALATES OF *Coptotermes formosanus*

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Abstract—Hexane extracts of the tergal glands from female alates of the Formosan subterranean termite *Coptotermes formosanus* were analyzed by high performance liquid chromatography—atmospheric pressure chemical ionization—mass spectrometry with collision-induced dissociation. Double bond configuration was determined by chemical modifications with gas chromatography—mass spectrometry. A single component, identified as the triacylglycerol, trilinolein, was unique to the female tergal glands. This compound was not found in other areas of the female alate abdomen or in the corresponding area of male alates. Neither gland extract nor trilinolein caused a behavioral response from male alates. However, significant differences were found between males and females for responses from neurons within sensilla of the maxillary palps.

Key Words—*Coptotermes formosanus*, Isoptera, Rhinotermitidae, Formosan subterranean termite, tergal gland, trilinoleoylglycerol, atmospheric pressure chemical ionization, mass spectrometry, sex pheromone.

INTRODUCTION

The use of semiochemicals by insects as a means of communication is well documented as are a multitude of exocrine glands, some of which are specialized for the production of pheromones (Noirot, 1969; Billen, 1994; Billen and Morgan, 1998).

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For subterranean termites (Rhinotermitidae), the use of chemical communication is a prominent means of expressing or eliciting social behaviors. In this family, the sternal gland is the major source of pheromones responsible for trail following and sexual attraction (Pasteels and Bordereau, 1998). However, the sternal glands in alates of the Formosan subterranean termite, *Coptotermes formosanus* Shiraki, are hypotrophied (unpublished observations). In conjunction with the fact that the females of this species do not exhibit calling to solicit mates it may be postulated that a sex pheromone, such as found in other species (Buchli, 1960; Leuthold, 1975), may be absent in *C. formosanus*. However, persistent maintenance of close contact by a male with the abdominal tip of the female during tandem behavior following swarming indicates the possible presence of a nonvolatile sex pheromone (Raina et al., 2003). The female alates of *C. formosanus* have a pair of tergal glands located beneath the 9th and 10th abdominal tergites, that are absent in the male (unpublished observation). Tergal glands have also been reported in several other species of termites (Noirot, 1969; Ampion and Quennedey, 1981), and in a few cases are implicated as a source of sex pheromone (Wall, 1971; Leuthold, 1975; Ampion, 1980). Recently, (*Z*,*Z*,*E*)-3,6,8-dodecatrien-1-ol, a chemical associated with the sternal glands of several termite species, was identified in volatile collections from tergal glands of *Cornitermes bequarerti* (Bordereau et al., 2002). In our study, we investigated the chemical composition of the tergal gland extract and tested the major component for sex-based behavioral activity and electrophysiological responses.

METHODS AND MATERIALS

Chemicals. OmniSolv HPLC grade hexane was acquired from EM Science (Gibbstown, NJ). HPLC grade methanol and methylene chloride were acquired from J. T. Baker (Phillipsburg, NJ). HPLC grade ethyl ether (unstabilized) was purchased from Burdick & Jackson (Muskegon, MI). Trilinolein, triolein, and tristearin were purchased from Sigma Chemical Co. (St. Louis, MO).

Insects. *C. formosanus* alates were collected from light traps placed at three locations in New Orleans, LA, from mid April until the first week of June 2001–2003. Samples were collected from the traps the following morning, sexed, and processed within 24 hr.

Instrumentation. HPLC–MS analyses were carried out with a Waters Alliance 2690 HPLC (Milford, MA), with column heater, 996 Photodiode Array Detector, and a Micromass ZMD (Waters, Milford, MA) or Waters Integrity TMD mass spectrometer (Milford, MA). A Luna silica (2), 5 μm, 2.0 $\times$ 150 mm (Phenomenex, Torrance, CA) column was used at a temperature of 35°C. The ZMD MS was equipped with an atmospheric pressure chemical ionization (APCI) source

and parameters set as corona voltage, 3.5 kV; cone voltage, 30 V; source block temperature, 125°C; APCI heater, 400°C; desolvation gas, 150 l/min; cone gas flow, 100 l/min; scan range, *m*/*z* 151–1000. Reversed phase LC–MS was performed using a Beckman HPLC with 126 pump, 168 PDA (Fullerton, CA), and a Thermoquest LCQ mass spectrometer (San Jose, CA). A Luna C18, 3 μm, 2.0 × 50 mm (Phenomenex, Torrance, CA) column was used at ambient temperature. The LCQ MS was equipped with an APCI source and parameters set as APCI vaporizer temperature, 450°C; source current, 5 μA; sheath gas flow, 80 au; auxilliary gas flow, 20 au; capillary temperature, 150°C; capillary voltage, 23 V; scan range, *m*/*z* 250–1500.

GC–MS was performed on an Hewlett-Packard 6890 GC and 5973 mass-selective detector (Agilent Technologies, Palo Alto, CA). Electron impact (EI) MS were obtained at 70 eV. A split/splitless injector was used in splitless mode with a purge flow to split at 2.0 min after injection. Chromatograms were run at a constant flow of 1 ml/min of He gas. The inlet temperature was set at 250°C. An HP-5MS (5% phenyl – 95% dimethylsiloxane) capillary column (30 m × 250 μm, 0.25 μm nominal) was used with temperature programming from 60°C (1-min hold) to 300°C at 10°C/min with a final 10-min hold. Samples were manually injected. Mass spectra were recorded from *m*/*z* 40 to *m*/*z* 550.

Extraction. The three terminal segments (8–10) from 50 female or male abdomens were excised and placed on filter paper. All the internal organs and fat bodies were gently removed with fine forceps, and the clean tips with tergal glands were extracted in 300 μl hexane for 1 hr. The hexane was removed, and the tips were rinsed again with 250 μl hexane. The combined hexane extract was concentrated with nitrogen to 100 μl. For individual tergal gland extractions, three terminal abdominal tergites from individual females were cut and placed in a drop of phosphate buffered saline (PBS, 0.01 M phosphate buffer, 0.0027 M potassium chloride, and 0.137 M sodium chloride, pH 7.4). All the internal organs and fat bodies were carefully removed. The area of the 9th and 10th tergites containing the pair of glands was excised and placed in a mixture of hexane, acetonitrile, and 2% NaCl (aq) (200:100:100 μl) and sonicated for 20 sec, × 3. The vial was centrifuged for 5 min at 2000 rpm. The hexane phase was removed and concentrated to 100 μl.

LC–MS Analysis. Normal phase analyses were performed on 100 μl injections. Injections of 50 termite equivalent samples (abdominal tip extract) were carried out with a 20-min gradient from 1% ether in hexane to 50% ether at a flow rate of 0.3 ml/min. The chromatography of the one equivalent samples (gland extract) was carried out with a 10-min ether/hexane gradient from 1 to 10% ether followed by a 2-min hold and a 5-min gradient to 50% ether at 0.5 ml/min (MS parameter changes: desolvation gas, 450 l/min; APCI heater, 500°C). Reversed phase analyses were performed on 10 μl injections (0.5 termite equivalents). The gradient used was 0–50% methylene chloride in methanol over 20 min at a flow

rate of 0.2 ml/min. Identification was confirmed with an authentic standard that had the same HPLC retention time and mass spectrum.

Double Bond Position Analysis. Tergal gland extract (9.5 termite equivalents) was purified by RP-HPLC using a water:methanol:ether gradient (30:70:0 to 0:100:0 in 15 min, 30-min hold, followed by a 20-min gradient to 0:80:20). The trilinolein fraction was concentrated with nitrogen to dryness. The residue was dissolved in 50 μl ether and 5 μl 0.5 M NaOMe/MeOH (Sigma, St. Louis, MO) were added. After mixing for 5 min, 5 μl 1% acetic acid were added to quench the reaction, and the ether solution was extracted with 25 μl saturated NaCl. The aqueous layer was removed and extracted with 25 μl ether. The combined ether layers were dried with anhydrous Na_2SO_4, transferred to a 1.5-ml vial, and evaporated to dryness with nitrogen. The fatty acid methyl esters (FAME) were analyzed by LC–MS (0.5 equivalent sample) and GC–MS (0.14 equivalent sample) (HPLC retention time, 15.5 min; *m*/*z* 317.3 (MNa^+). GC retention time, 18.92 min; mass spectrum matches methyl linoleate standard). The FAME residue was dissolved in hexane (200 μl) and 200 μl dimethyl disulfide (Aldrich, Milwaukee, WI), and 100 μl I_2/ether (60 mg/ml) were added. The mixture was heated in a sand bath at 50°C for 72 hr. After cooling to room temperature, the reaction mixture was diluted with 400 μl hexane and extracted with 300 μl 5% sodium thiosulfate. The clear hexane solution was dried (anhydrous Na_2SO_4), filtered, and concentrated to dryness with nitrogen. The residue was dissolved in 10 μl hexane. A 0.9 equivalent sample was analyzed by GC–MS (retention times, 24.78, 27.11, 27.48, 27.59, and 27.66 min; mass spectra match DMDS products from methyl linoleate standard).

Behavioral Bioassays. Hexane extract of the female abdominal tip was applied to an 8 × 1.5 mm Teflon coated magnetic stir bar (VWR Scientific). The stir bar was placed on a filter paper in a 90 × 20 glass Petri dish. A dealate male was released into the Petri dish, and the stir bar was moved along the edge with the help of a magnet held on the outside. Male behavior was observed for 2 min. The test was repeated ×5. Modifications to the assay were tested by replacing the stir bar with a female dealate cadaver or live male dealates, and by replacing the hexane extract with synthetic trilinolein.

Electrophysiological Recordings. Electrical responses of neurons associated with the long trichoid chemosensilla on antennae and maxillary palps of male and female *C. formosanus* alates were recorded by using a standard tip-recording technique (Hodgson et al., 1955). Adult termites were affixed inverted on cork and were held in place with tape. Antennae were pulled forward and immobilized with two-sided sticky tape for easy access to sensilla on the terminal antennal segment. Maxillary palps were similarly immobilized for access to sensilla at the tip. A sharpened tungsten electrode inserted into the abdomen of the insect served as a reference electrode for all preparations. The stimulating/recording electrode comprised a silver wire inserted into a glass capillary, pulled and sized to fit over the tip of a single chemosensory hair. This electrode was filled with

the test chemicals in a 0.01 M NaCl solution (90% distilled water and 10% ethyl acetate). Electrical activity was amplified and conditioned with a Grass P15D amplifier, viewed on an oscilloscope, monitored with a loudspeaker, and digitized for storage and analysis on a lab computer using Sapid (Smith et al., 1990) and AutoSpike (Syntech, Hilversum, The Netherlands) software. Mean numbers of spikes for various stimuli were compared by using a two-tailed *t* test for unequal sample sizes (Ostle, 1963). Because of its nonpolarity and low solubility in water, trilinolein was solubilized in ethyl acetate prior to mixing with the electrolyte solution (10 mM NaCl). The presence of trilinolein in solution in ethyl acetate was verified with HPLC. To observe its presence in the final electrophysiological stimulating solution, a small amount of 10 mM trilinolein in 10 mM NaCl (10% ethyl acetate) from a capillary tube was spotted on a TLC plate, stained with PMA reagent, and charred on a hot plate. This revealed a dense spot relative to a 10 mM NaCl (10% EtOAc) control, which did not react with PMA reagent.

Responses were obtained from 1–5 chemosensory hairs for at least three males and three females for the following treatment chemicals: 10 mM NaCl (10% ethyl acetate) and 10 mM trilinolein in 10 mM NaCl (10% ethyl acetate). Individual hairs were stimulated with trilinolein, followed by 10 mM NaCl (10% ethyl acetate), and then restimulated with the trilinolein solution to confirm electrical responses of neurons for one male maxillary palp preparation, two male antennal preparations, and four female antennal and maxillary palp preparations.

Nerve impulses within the first 500 msec following stimulation were counted. Statistical analyses were conducted using each insect as the experimental unit, averaging nerve impulse counts over all hairs for a given insect. The average numbers of spikes observed for each insect for the two treatments were compared using a two-tailed *t* test for unequal sample sizes (Ostle, 1963).

RESULTS

Chemical Analysis of Tergal Gland Extracts. A pair of tergal glands was observed under the 9th and 10th abdominal tergites of the female (Figure 1A) that was absent in the male alates. At the ultrastructural level, the gland cells contained a large number of electron dense lipoidal granules (Figure 1B). The three terminal segments (8–10) of both male and female abdomens were extracted with hexane and analyzed using normal phase (Figure 2A) and reversed phase (Figure 2B) chromatography with APCI–MS detection. All peaks in the chromatograms of the male extract were also present in the female extract. However, a unique peak was observed in the chromatogram of the female extract with a retention time of 11.1 min (normal phase) or 13.5 min (reversed phase). This peak was absent in extracts of the remaining part (segments 1–7) of the female abdomen. The major peaks observed from the male and female alate tip extracts had retention times

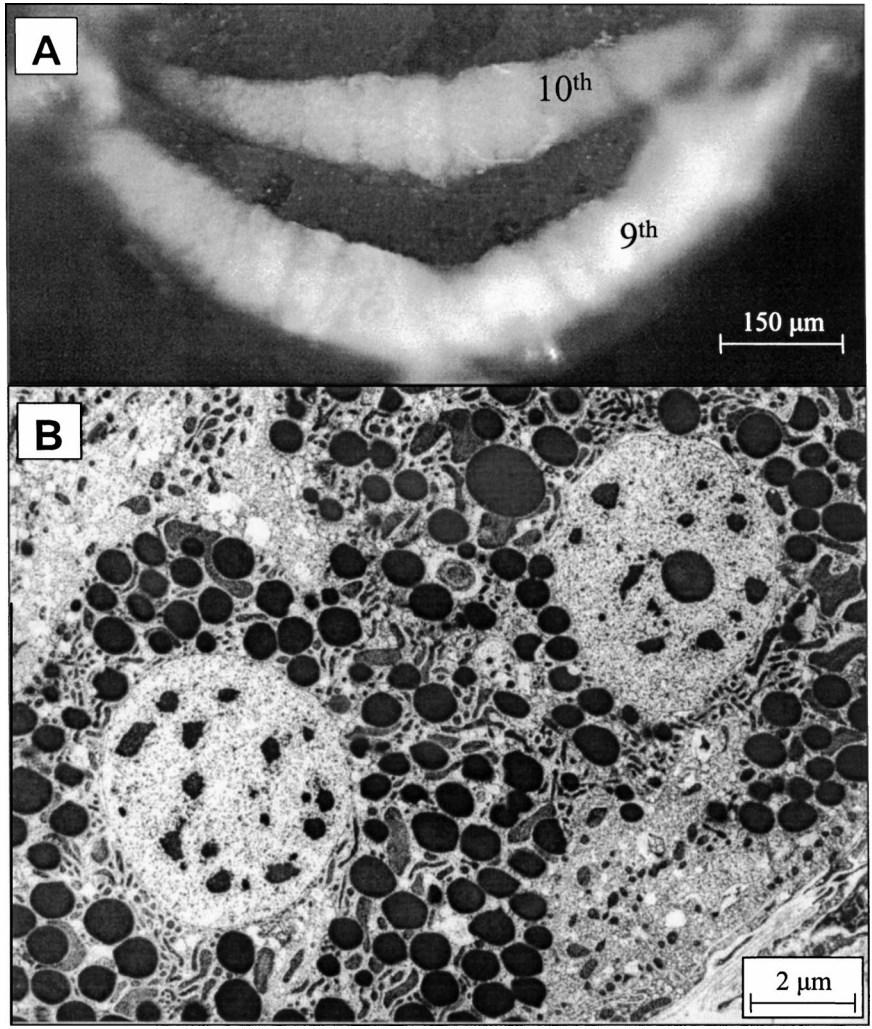

FIG. 1. Tergal glands of female *C. formosanus*. (A) Photograph of the pair of glands located under the 9th and 10th abdominal tergites. (B) Electron micrograph of thin section through portion of a tergal gland showing large number of electron dense lipoidal granules in the cytoplasm of the cells.

(10–11 min) in the normal phase chromatogram that corresponded to triacylglycerol (TAG) standards. The retention times of TAGs with the same total number of carbons in the three fatty acids (CN) were proportional to the total number of double bonds (ND) as shown in Table 1, or inversely proportional to the molecular

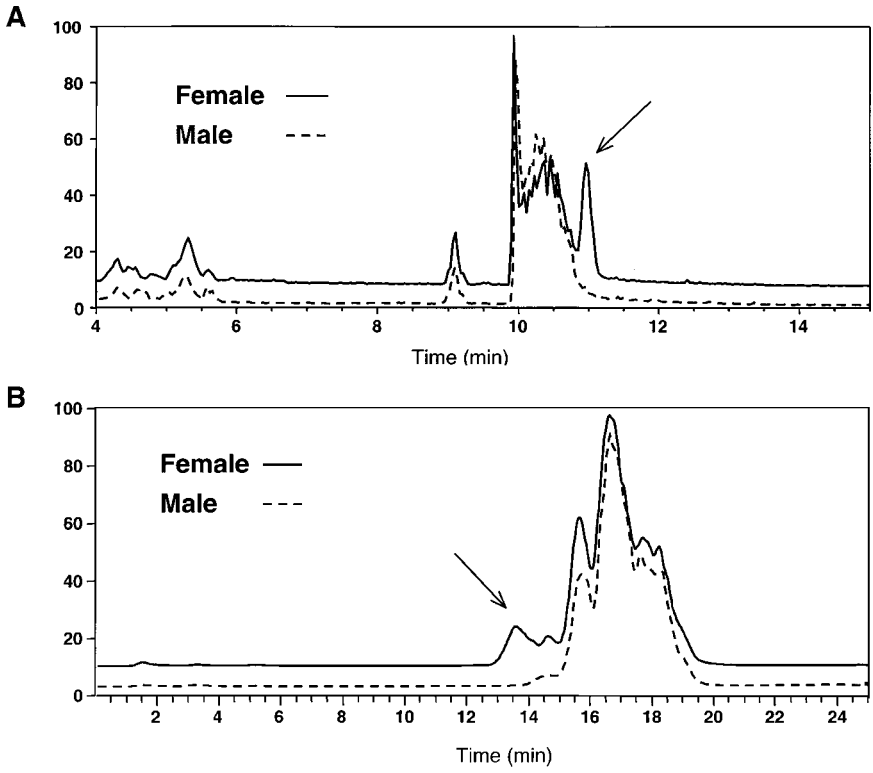

FIG. 2. HPLC–APCI–MS chromatogram of the hexane extract of abdominal segments 8–10 of *C. formosanus* male and female alates: (A) normal phase; (B) reversed phase. Trilinolein peak indicated with arrow.

weight. The protonated forms (MH^+) of the TAGs containing stearic (S), oleic (O), and linoleic (L) fatty acids give rise to the masses shown in Table 1. The masses found in the male extract included all possible masses from 891 to 881, while the female had an additional 879 mass. As seen in Table 1, this indicated that gland extracts of females contained a compound whose characteristics resembled those of trilinolein (LLL) that was absent in the male extract. Reversed phase chromatography showed the reverse pattern.

In addition to the MH^+ values found for each TAG in the APCI–MS spectra, fragmentation peaks corresponding to the loss of the 1- or 3-fatty acids were also observed (Kusaka et al., 1996). In fact, for the more saturated triacylglycerols, the diacylglycerol (DAG) fragment resulting from the loss of the fatty acid is more prominent than the parent mass. Table 2 shows the masses and DAG fragments resulting from the loss of the 1- or 3-fatty acid. As with a trilinolein standard, the

TABLE 1. TRIACYLGLYCEROLS (TAG) IN *C. Formosanus* ALATE CUTICLE TIP EXTRACT

TAG[a]	CN[b]	ND[c]	MH^+	RT-N[d]	RT-RP[e]	Male	Female
S,S,S	54	0	891	9.82	19.1	+	+
S,S,O*	54	1	889	9.82	18.3	+	+
S,O,O*; S,S,L*	54	2	887	9.84	17.6	+	+
O,O,O; S,O,L*	54	3	885	10.64	16.7	+	+
O,O,L*; S,L,L*	54	4	883	10.80	15.6	+	+
O,L,L*	54	5	881	11.01	14.7	+	+
L,L,L	54	6	879	11.11	13.5	−	+

Note. TAG with * contains multiple positional isomers.
[a] Triacylglycerol fatty acids are designated as S (stearic), O (oleic), and L (linoleic). TAGs identities based on SSS, OOO, and LLL standards; other TAGs tentatively identified by MS MH^+ and fragmentation.
[b] Number of carbon atoms in the three fatty acids.
[c] Number of double bonds in the three fatty acids.
[d] Normal phase chromatography retention time (min).
[e] Reversed phase chromatography retention time (min).

unique peak found in the female tergal gland displayed only the fragment ion of m/z 599 (Figure 3), resulting from the loss of one linoleic acid.

The fragmentation of both the 1- and 3-fatty acids (as shown in Table 2) produces an ion that identifies the fatty acid in the 2-position. As noted above, as the TAG became more unsaturated, less fragmentation was observed. With trilinolein standard, the ion of m/z 337 was observed only for high concentrations. The fragmentation could be increased with in-source collision-induced dissociation (CID), causing the 2-monoacylglycerol (MAG) fragment to be more prominent. The ion of m/z 337 was the only MAG fragment ion observed (Figure 3) associated with the female-specific HPLC peak. Only trilinolein (trilinoleoylglycerol) can match this pattern of parent and fragment masses, verifying it as the structure of the female-specific tergal gland component.

The positions of the double bonds in the triglyceride were verified by formation of the fatty acid methyl esters (FAME) and subsequent derivatization with dimethyl disulfide (DMDS). Both the FAME and DMDS products obtained from the tergal gland extract component coincided in HPLC or GC retention time and mass spectra with the same products from authentic trilinolein.

Quantification of Trilinolein. For quantification purposes, the tergal glands from a single female, with as little extraneous tissue as possible, were extracted. The trilinolein peak comprised approximately 88% of the total peak area from the extract. On the basis of a standard curve for trilinolein, the amount of trilinolein found in the female alate tergal gland was 845 ± 205 ng (mean $\pm$ SD, $N = 6$). This level was dependent on the extraction method. A 1–2 hr hexane extraction at room temperature resulted in the isolation of small amounts of trilinolein, on the order of 291 ± 261 ng. Overnight extraction at 4°C increased the amounts slightly,

TABLE 2. APCI–MS FRAGMENT MASSES OF *C. FORMOSANUS* ALATE TRIACYLGLYCEROLS (TAG); DIACYLGLYCEROL (DAG; MH^+-RCO_2H) AND MONOACYLGLYCEROL (MAG; MH^+-RCO_2-RCO) FRAGMENTS

		DAG		MAG	
TAG	MH^+ (m/z)	Fragments[a]	m/z	Fragments[a]	m/z
SSS	891	SSx	607	xSx	341
SSO	889	SSx, OSx	607, 605	xSx	341
SOS	889	SOx	605	xOx	339
SOO	887	SOx, OOx	605, 603	xOx	339
OSO	887	OSx	605	xSx	341
SSL	887	SSx, LSx	607, 603	xSx	341
SLS	887	SLx	603	xLx	337
OOO	885	OOx	603	xOx	339
SOL	885	SOx, LOx	605, 601	xOx	339
OSL	885	OSx, LSx,	605, 603	xSx	341
SLO	885	SLx, OLx	603, 601	xLx	337
OOL	883	OOx, LOx	603, 601	xOx	339
OLO	883	OLx	601	xLx	337
SLL	883	SLx, LLx	603, 599	xLx	337
LSL	883	LSx	603	xSx	341
LOL	881	LOx	601	xOx	339
OLL	881	OLx, LLx	601, 599	xLx	337
LLL	879	LLx	599	xLx	337

[a] Fragments shown with x denoting position of lost fatty acid. Fatty acids designated as described in Table 1.

but by sonicating the tissue for 1 min in a 2:1:1 mixture of hexane/acetonitrile/2% NaCl, as much as 1136 ng of trilinolein were extracted into the hexane phase from the tergal glands of a single female.

Behavioral Bioassays. Males touched the hexane extract coated magnetic stir bar with their antennae, but the contact was very brief. After the first contact, there were no further attempts to touch the stir bar. Similar results were obtained from the modifications to the assay, with some degree of repellency observed when a female cadaver was used in the test.

Electrophysiological Recordings. Tip recordings from long trichoid sensilla on the maxillary palps of male *C. formosanus* revealed differential responses of neurons to experimental stimuli. While responses to both the solvent control 10 mM NaCl (10% ethyl acetate) and 10 mM trilinolein were generally multineuronal, activity of a single spike height was observed in some sensilla. Repeated stimulation with trilinolein elicited activity of a single spike height with consistent interspike intervals in male maxillary palps, while responses to the solvent control elicited little activity in males or multiple spike heights in female maxillary palp preparations (Figure 4). Mean numbers of spikes elicited by 10 mM trilinolein were

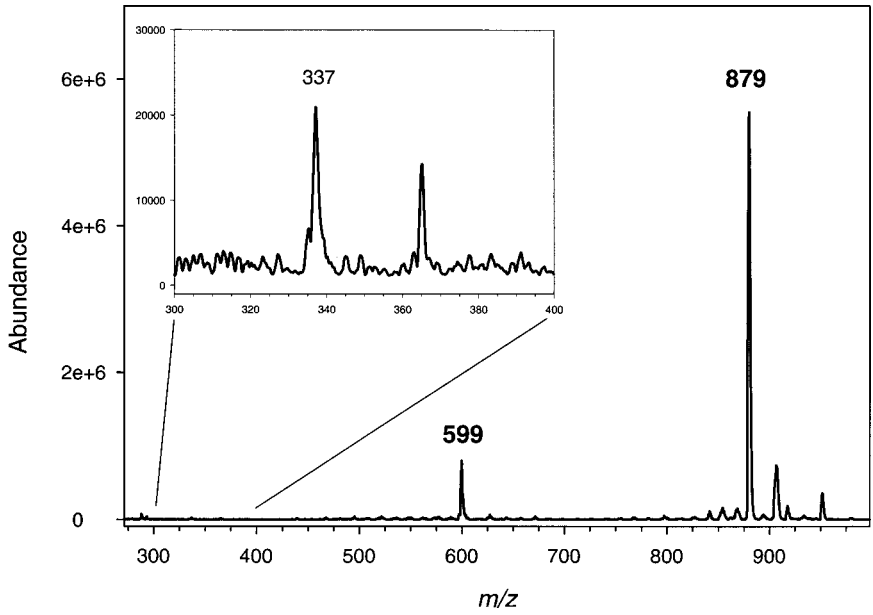

FIG. 3. APCI–MS of tergal gland component (normal phase HPLC 11.1 min peak) showing ions specific for trilinolein.

greater than numbers of spikes elicited by the control for both maxillary palp and antennal preparations in males. For maxillary palps, these differences were significant ($P < 0.05$, two-tailed t test for unequal sample sizes; Ostle, 1963) (*male maxillary palp*: trilinolein 30.1 ± 4.5 spikes/initial 500 msec ($N = 7$ sensilla on three males), control 3.3 ± 2.9 spikes/initial 500 msec ($N = 7$ sensilla on three males); *male antenna*: trilinolein 19.8 ± 9.1 spikes/initial 500 msec ($N = 9$ sensilla on three males), control 8.9 ± 5.3 spikes/initial 500 msec ($N = 9$ sensilla on three males)).

There were no significant differences in the number of spikes recorded from female antennal or maxillary palp sensilla to 10 mM trilinolein vs. solvent control (*female antenna*: trilinolein 10.0 ± 1.5 spikes/initial 500 msec ($N = 21$ sensilla on six females), control 7.8 ± 0.5 spikes/initial 500 msec ($N = 21$ sensilla on six females); *female maxillary palps*: trilinolein 24.6 ± 0.7 spikes/initial 500 msec ($N = 2$ sensilla on four females), control 29.4 ± 5.7 spikes/initial 500 msec ($N = 12$ sensilla on four females)).

Female extract showed a significant difference in the number of spikes recorded for the male antennae and maxillary palps at $P < 0.05$ (two-tailed t test for unequal sample sizes; Ostle, 1963) (*male antenna*: female extract 16.0 ± 2.4 spikes/initial 500 msec ($N = 27$ sensilla on 9 males), control 9.2 ± 1.6 spikes/initial 500 msec ($N = 29$ sensilla on 11 males); *male maxillary palps*:

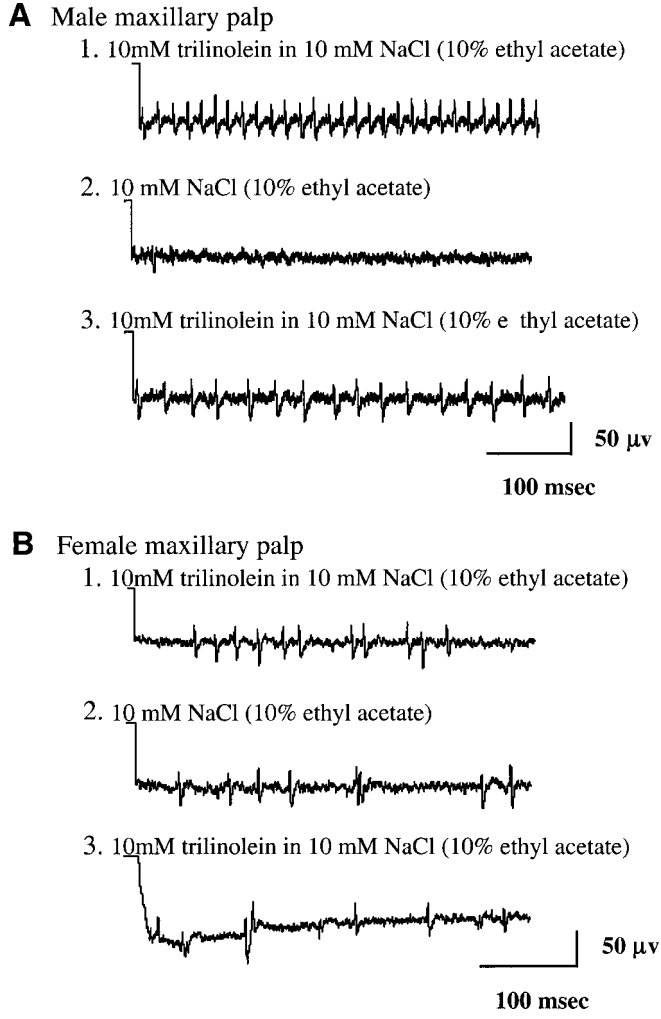

FIG. 4. Electrical activity of neurons housed within two long trichoid sensilla near the tip of a maxillary palp of male (A) and female (B) *Coptotermes formosanus* alate. (A) 1: Initial response to trilinolein (10 mM); 2: response to solvent control (0.01 M NaCl); 3: response to trilinolein (10 mM) after control. Each trace represents 500 msec following contact of electrode with tip of sensillum.

female extract 21.4 ± 2.0 spikes/initial 500 msec ($N = 18$ sensilla on 5 males), control 13.8 ± 2.1 spikes/initial 500 msec ($N = 36$ sensilla on 9 males)).

There were no significant differences in the number of spikes recorded from female antennal or maxillary palp sensilla to female extract vs. solvent control

(*female antenna*: female extract 8.1 ± 2.3 spikes/initial 500 msec ($N = 7$ sensilla on three females), control 13.9 ± 3.7 spikes/initial 500 msec ($N = 7$ sensilla on three females); *female maxillary palps*: female extract 16.7 ± 3.0 spikes/initial 500 msec ($N = 10$ sensilla on three females), control 22.4 ± 1.3 spikes/initial 500 msec ($N = 10$ sensilla on three females)).

DISCUSSION

In most termite species that exhibit calling behavior, the sex pheromone involved has been identified as (*Z*, *Z*, *E*)-3,6,8-dodecatrien-1-ol (DTE-OH), the same chemical as the trail pheromone, originating in the sternal glands of workers (Matsumura et al., 1968; McDowell and Oloo, 1984; Bordereau et al., 1993; Laduguie et al., 1994; Wobst et al., 1999). In cases where it has been quantified, the amount of DTE-OH in the sternal gland of the female alate is approximately 10 times that of the male alate and 10–2000 times that of the worker. The trail pheromone of *C. formosanus* workers originating from the sternal glands has also been identified as DTE-OH (Matsumura et al., 1972; Tokoro et al., 1989, 1992). However, DTE-OH has not been reported from the alates of this species.

C. formosanus female dealates do not exhibit a calling behavior, and the males are not attracted to a female even from a close distance (Raina et al., 2003). It was also observed that the males form tandem pairs only after coming into contact with a female. In addition, *C. formosanus* alates have hypotrophied sternal glands compared to workers (unpublished observations). However, the females do possess a pair of tergal glands under the 9th and 10th abdominal tergites that are not present in the male alates (unpublished observations) similar to several other species (Noirot, 1969; Ampion and Quennedey, 1981). Buchli (1960) speculated that for species exhibiting calling behavior, a separate chemical from that used to initiate attraction may be responsible for tandeming behavior. Noirot (1969), Leuthold (1975), and McDowell and Oloo (1984) suggested the production of a sex pheromone by the tergal glands. Barth (1955) observed in *Syntermes dirus* and Leuthold (1975) in *Trinervitermes bettonianus* that varnishing of the last abdominal segments of the female eliminated tandem behavior.

Our results show that the tergal glands in females of *C. formosanus* contain the triacylglycerol trilinolein. This compound was not found in other areas of the female or in the male dealate. Trilinolein comprised as much as 88% of the triglycerides extracted from tergal glands uncontaminated by extraneous fat bodies. Extraction of trilinolein was method-dependent, with whole body extractions producing only small amounts of trilinolein. Use of sonication in the extraction procedure increased its recovery, indicating that the compound may be sequestered within the tergal gland as compared to volatile pheromones such as

DTE-OH. The amount of trilinolein in a single *C. formosanus* female alate was about 850 ng (range, 2–1136 ng). The large variability could be a natural phenomenon that may contribute to mate selection, or it may be due to differences in the physiological age of the female following the last molt. On the whole, the average amount of trilinolein falls between that of DTE-OH (1–20 ng in *R. santonensis* or *P. spiniger* alates; Bordereau et al., 1991; Laduguie et al., 1994), and cembrene-A (12 μg in *T. bettonianus* female alates; McDowell and Oloo, 1984).

To determine the possible behavioral role of trilinolein, we conducted several bioassays for tandem behavior with both female tergal gland extract and authentic trilinolein. However, neither evoked a tandem response from dealate males. Although brief contacts were established with a treated surface, the behavior did not persist for long. It is possible that the male may be using tactile cues from a female in addition to a chemical cue from trilinolein. Another possibility is that trilinolein may be a precursor to a biologically active compound, as has been shown by Tokoro et al. (1992) in the case of DTE-OH. However, since abdominal tip extracts also did not yield positive results in the bioassays, an active substance may not be present or required in a concentration range not tested, or the bioassay methods may be inadequate to test for this behavior.

Electrophysiology studies demonstrated the effect of trilinolein or tergal gland extract on sensilla present on the maxillary palps and antennae of the male and female alates. Previous observations of tandem behavior had revealed that male antennae play an important role in locating a female whereas maxillary and labial palps are involved in maintaining a constant contact with the tip of female abdomen (Raina et al., 2003). Extract of the female abdominal tip elicited significantly higher spike activity as compared to control, in sensilla on both the maxillary palps and antennae of the male. In the female, differences in response were not significant. Trilinolein elicited a similar response, but significant only for sensilla on the maxillary palps. The observed neural activity indicates detection of trilinolein by male alates only, which suggests its involvement in some type of female–male chemical communication. There are indications that trilinolein may act as a nuptial gift from female to male. This aspect needs further investigation. In conclusion, trilinolein was identified as the major component in extracts of the tergal glands of female alates of *C. formosanus*, having female specificity in its production and male specificity in its perception.

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COMPOSITION OF CHEMICAL ATTRACTANTS AFFECTS TRAP CATCHES OF THE AUSTRALIAN SHEEP BLOWFLY, *Lucilia cuprina*, AND OTHER BLOWFLIES

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Abstract—Numbers of *Lucilia cuprina* (Australian sheep blowfly), *Chrysomya* spp., and *Calliphora* spp. blowflies caught on sticky traps baited with various synthetic attractants or a standard liver/sodium sulfide attractant in western Queensland were recorded. Numbers of each genus collected were influenced by the composition of the chemical attractants. Attractant mixtures based on 2-mercaptoethanol, indole, butanoic/pentanoic acid, and a sodium sulfide solution gave 5- to 20-fold higher *L. cuprina* catches than the liver standard. These blends attracted similar numbers of *Chrysomya* spp. (0.85–2.7×) and fewer *Calliphora* spp. (0.02–0.2×) compared to the liver standard. These synthetic attractants were more effective and selective for *L. cuprina* than the standard liver/sodium sulfide attractant, and they can be packaged in controlled-release dispensers to generate constant, prolonged release of the attractant.

Key Words—Australian sheep blowfly, *Lucilia cuprina*, field trials, kairomones, chemical attractants, sticky traps, attractancy, selectivity.

INTRODUCTION

The Australian sheep blowfly, *Lucilia cuprina*, initiates more than 85% of fly strikes (cutaneous myiasis) in Australia (Anderson et al., 1988). The estimated average annual cost of blowfly control and production loss for the Australian wool industry is A$160 million (McLeod, 1995). Current control of blowfly strike,

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aside from good husbandry and management, relies primarily on the application of insecticides. Insecticidal control is becoming increasingly problematic due to the presence of chemical residues in fleece and the resistance of blowflies toward many insecticides (Levot, 1995).

It has been shown that fly density and strike incidence can be reduced by blowfly trapping. For example, Mackerras (1936) used a grid of blowfly traps (Western Australian traps and Meteor traps) on properties near Canberra to achieve a 57% reduction in the strike rate compared to the control properties (five experiments). More recently, the placement of bait-bins on a sheep property with quantitative historical records of fly numbers and strike incidence indicated that blowfly strike and *L. cuprina* numbers were lowered by their presence (Anderson, 1990; Anderson et al., 1990; Cook, 1990). The trapping systems used in both these studies were cumbersome, uneconomical, and probably inefficient in catching *L. cuprina*. The development of a practical, effective, and economical trapping system for the Australian sheep blowfly could make fly population and strike incidence suppression a feasible control strategy. Such control methods could reduce insecticide usage and decrease associated residues in products from sheep, leading to improved animal welfare and human health.

An effective and selective attractant for *L. cuprina* is an essential component for such a system. The established attractant used for the Australian sheep blowfly is liver, made more attractive by the addition of an aqueous sodium sulfide solution (Freney, 1937; Vogt and Havenstein, 1974). The variability of this attractant over time (Vogt and Woodburn, 1994), the frequent servicing required to keep the attractant active (Vogt and Morton, 1991), and between-batch variability makes this a less than ideal standard attractant. An effective synthetic attractant packaged in a controlled-release dispenser would be a distinct improvement. Studies over the last 60 years have focussed on identification of volatile components emitted by natural sources that attract sheep blowflies. The early work has been reviewed (Eisemann, 1985; Ashworth and Wall, 1994), and more recent studies have been carried out on the orientation of *L. cuprina* toward fleece and chemical attractants (Eisemann, 1995; Morris et al., 1998), and on the identification of volatile components from bacterial strains isolated from myiatic lesions of sheep (Khoga et al., 2002).

One of the promising leads in sheep blowfly attractants research came from Eisemann (1995), who demonstrated attraction in a laboratory assay of *L. cuprina* to binary and tertiary mixtures composed of 2-mercaptoethanol, indole, and hydrogen sulfide. Volatile fatty acids, present in wool wax and produced by *Pseudomonas aeruginosa* (Labows et al., 1980) were also likely candidate attractants. *P. aeruginosa* is a causative agent of fleece rot, which is a predisposing condition of fly strike (Gherardi et al., 1981).

Similar investigations of attractants for the New World screwworm fly, *Cochliomyia hominivorax*, resulted in the development and successful application

of synthetic Swormlure attractants (Coppedge et al., 1977; Mackley and Brown, 1984). These attractants and several modified mixtures were also included in this study of potential sheep blowfly attractants.

Assessment of *L. cuprina* responses to olfactory stimuli has generally been carried out with laboratory fly strains under laboratory or insectary conditions (Eisemann, 1985, 1995; Ashworth and Wall, 1994; Urech et al., 1994; Morris et al., 1998). Field assessments are required for two reasons. First, laboratory results are not always reliable indicators of field performance, and second, the selectivity of the attractants for the target species needs to be assessed. Mackerras (1936) stated that new blowfly control measures had to be directed specifically against *L. cuprina*, without unduly affecting other species that are potent natural agents in limiting the abundance of *L. cuprina* and other primary flies. Endemic Australian blowflies, in particular *Chrysomya rufifacies*, outcompete *L. cuprina* on carcasses (Kitching, 1981; Cook et al., 1995) and, thus, limit the population growth of the Australian sheep blowfly. Because these native flies rarely initiate strikes in Australia, it is beneficial for them to remain in the ecosystem. A selective attractant also lessens saturation of traps with nontarget species.

To evaluate the performance of synthetic attractants in the field, a trap was needed that did not discriminate between fly species. Sticky traps are more efficient in catching insects than restricted entry/exit traps, because all insects which land are retained (Spradbery, 1981; Muirhead-Thomson, 1991). In addition, Wardhaugh et al. (1984) used sticky traps to determine spatial distributions of *L. cuprina.*

Herein, we report the performance of selected synthetic attractants as baits in sticky traps placed in typical sheep-grazing areas of western Queensland, Australia. Chemical mixtures evaluated in these trials comprised components tested previously in laboratory systems (Eisemann, 1985, 1995; Ashworth and Wall, 1994; Urech et al., 1994), and of modified Swormlure formulations (Coppedge et al., 1977; Mackley and Brown, 1984). The effect of the composition of these attractants on field catches of *L.cuprina* and other blowflies is reported here. Part of this work has been reported previously (Urech et al., 1993).

METHODS AND MATERIALS

Field trials were conducted between September 1990 and March 1992 on sheep properties in western Queensland, Australia. The objective was to obtain direct comparisons of the efficacy of different attractants for the various fly species present on sheep properties. All trials used a Latin square design (Perry et al., 1980).

A widely used standard sheep blowfly attractant consisting of liver and a sodium sulfide solution (Mackerras et al., 1936; Vogt and Havenstein, 1974) was used as the reference attractant. The performance of the synthetic attractants was

compared with this standard, which served as an indicator of the density and composition of blowfly populations. The performance of an attractant was described by its attractive potency for various blowfly genera, and its selectivity for *L. cuprina* was compared to the other genera (terms defined below).

Traps and Attractants. A modified sticky trap (Spradbery, 1981; Wardhaugh et al., 1984) with a galvanized sheet metal base (250 × 250 mm) and a flat roof (300 × 300 mm, 130 mm above base) was used. Below the base were two centrally located wells (diam 30 mm) for attractant bottles, and a vertical, round metal tube (150 mm long, 50 mm diam) that allowed the trap to be placed on a steel post, with the base 1.2 m above ground. An interchangeable polyethylene tray (250 × 250 mm), coated over a 200 × 200 mm square with Tanglefoot (The Tanglefoot Company, Grand Rapids, MI 49504), covered the base. One or two glass bottles (28 ml McCartney) containing the attractant mixtures (10 ml) were placed in the trap wells, with the bottle top (internal diam 20 mm) extending just above the polyethylene tray. The compositions of the attractant mixtures and the corresponding codes are given in Table 1 (each X_n represents a separate bottle). The H series were aqueous solutions of sodium sulfide (Ajax Chemicals, Auburn NSW 2144, technical, 68%) that, when exposed to the atmosphere, released hydrogen sulfide. The M attractants all contained 2-mercaptoethanol, a previously identified sheep blowfly attractant (Eisemann, 1985). The N series mixtures were related to Swormlure-2 (Coppedge et al., 1977). All components were of analytical or laboratory reagent grade and purchased from Sigma–Aldrich (Castle Hill, NSW 1765) or Ajax Chemicals. A filter paper strip, 75 mm high (equal to height of bottle) and 15 or 60 mm wide (folded into a "W" shape for the latter) was placed in the M and N bottles. The wider strip provided improved evaporation at lower temperatures. The standard liver/sodium sulfide attractant (S) was freshly prepared 2–3 d prior to each experiment, so that its day degree age was 50–100 (Vogt and Woodburn, 1994) at the start of the experiment. A standard fly mesh was draped over the S bottle to prevent fly entry and egg laying on the liver. All bottles were used for one experiment only.

Experimental Procedure. The experimental designs were Latin squares (LS) (Perry et al., 1980), either a duplicated 4 × 4 LS with four attractants over 4 d with eight trap sites, or a 5 × 5 LS with five treatments over 5 d and with five trap sites. Trap sites were at least 100 m apart. Traps were located in shaded positions in preferred blowfly habitats such as sheep camps or near watering points. Comparable sites were chosen for each experiment. Each 24 hr the attractants were rotated, by random assignment, to new sites within the Latin square and fresh sticky trays were installed. The flies from the harvested trays were counted and categorized as female *L. cuprina*, male *L. cuprina*, *Chrysomya* spp., *Calliphora* spp., and other flies. A subsample of 500 *Lucilia* spp. from trials in all locations was inspected for *L. sericata* (Holloway, 1991).

TABLE 1. COMPOSITION AND CODES OF SYNTHETIC ATTRACTANTS AND LIVER STANDARD (S)

	Attractant codes																
Components	S	H_1	H_2	M_1	M_2	M_3	M_4	M_5	M_6	M_7	M_8	M_9	M_{10}	N_1	N_2	N_3	N_4
Liver (g)	10																
Sodium sulfide (g)	0.15	4.5	0.45														
Water (ml)	10	10	10		5	4	4	9	8.8	8.8	8.8						
2-Mercaptoethanol (ml)				5	5	5	5	1	1	1	1	8	2				
Indole (g)					0.5	0.5	0.5	0.1	0.1	0.1	0.1	1	1	0.33	0.33	0.33	0.33
Pentanoic acid (ml)						1			0.2					1.2	1.2	1.2	1.2
Thiolacetic acid (ml)							1			0.2							
Butanoic acid (ml)											0.2	2	8	1.6	1.6	1.6	1.6
Phenol (g)														0.33	0.33	0.33	
4-Methylphenol (g)														0.33	0.33	0.33	
2-Methyl-1-propanol (ml)														0.84	0.84	0.84	0.84
2-Butanol (ml)														1.2	1.2	1.2	1.2
Acetone (ml)														0.75	0.75	0.75	0.75
Benzoic acid (g)														0.33	0.33	0.33	
Dimethyl disulfide (ml)														1.3	0.13	0.013	0.13
Acetic acid (ml)														1.2	1.2	1.2	1.2

The numbers of flies caught were transformed (square root $X + 0.5$) and subjected to analysis of variance with a comparison of mean catches using the protected LSD procedure at the 5% level of significance as described in the Latin square analysis by Perry et al. (1980). Transformation was required to normalize the data, and a square-root transformation was chosen since it provided the smallest coefficient of variance in most experiments. The data were back-transformed ($X^2 - 0.5$) for presentation in tables to provide a realistic measure of mean daily fly catches used for calculation of the following performance characteristics:

Percent L. cuprina out of total *Lucilia* + *Chrysomya* + *Calliphora* spp. trapped.

Percent male L. cuprina out of total *L. cuprina* trapped.

Potency of attractant for genus X = mean X caught on attractant/mean X caught on standard.

Selectivity of attractant for *L. cuprina* compared with genus Y = potency (*L. cuprina*)/potency (Y).

A potency value could not be calculated when the attractant and/or the standard catch was zero.

Effects of Modifications to Individual Attractant Components. These trials were designed to demonstrate the effect of adding/removing one component or altering its concentration on the fly catch. Such modifications in the M and N series were carried out in experiments 1–7 and 8–12, respectively. The results of these individual Latin square experiments are shown in Tables 2 and 3.

Comparison of M and N Series (Table 4). Results from the 12 trials reported in Tables 2 and 3 and from additional field trials (individual results not reported) were pooled to provide the best possible characterization of the M and N attractant series. The M series, based on 2-mercaptoethanol/indole/acid, included results from trials comprising M_3, M_6, and M_8–M_{10}. The N series included results from all Swormlure-based attractants N_1–N_4 (*cf.* Table 1). Both the M and N series were used in combination with an H series attractant. The means of the field experiments reported in Table 4 are back-transformed means (square root (X + 0.5) transformation) of the results (mean daily catch, % fly species and sex, potency, selectivity) obtained in all field tests involving attractants belonging to either of the two series. Results with zero fly counts for the attractant and standard, or where the divisor in formulas was zero, were excluded.

RESULTS AND DISCUSSION

Results from individual Latin square experiments examining the effects of various components on attractant performance are presented in Table 2 for mixtures based on 2-mercaptoethanol (2-me)/indole/hydrogen sulfide (H_2S) and in Table 3 for Swormlure-2-based mixtures (Coppedge et al., 1977). The mean daily fly catches for the three blowfly genera, the percentage of *L. cuprina* in the total

TABLE 2. BACK-TRANSFORMED MEAN DAILY FLY CATCHES AND PERFORMANCE CHARACTERISTICS FOR ATTRACTANTS BASED ON 2-MERCAPTOETHANOL/INDOLE/H_2S AND LIVER STANDARD (S)

		Mean daily catch[a]				Potency			Selectivity	
Experiment	Attractant	*L. cuprina*	*Chrysomya* spp.	*Calliphora* spp.	% *L. cuprina*	*L. cuprina*	*Chrysomya*	*Calliphora*	*L. cuprina*/ *Chrysomya*	*L. cuprina*/ *Calliphora*
1	H_1M_2	0.28^{B}	0.10	0.10^{C}	59.1	0.09	0.5	0.01	0.19	13.4
	H_1M_4	0.45^{B}	0	$0.88^{B,C}$	33.9	0.15	—	0.06	—	2.4
	H_1M_6	22.8^{A}	0.77	4.5^{B}	81.3	7.3	3.9	0.31	1.9	23.7
	S	3.1^{B}	0.20	14.4^{A}	17.5	1.0	1.0	1.0	1.0	1.0
2	H_1M_5	$1.2^{B,C}$	0	0.53^{B}	69.4	0.16	—	0.03	—	6.6
	H_1M_7	0.39^{C}	0.20	1.6^{B}	17.7	0.05	0.17	0.08	0.31	0.70
	H_1M_3	18.3^{A}	0.28	3.5^{B}	83.0	2.5	0.23	0.17	10.5	15.3
	S	$7.3^{A,B}$	1.2	21.1^{A}	24.7	1.0	1.0	1.0	1.0	1.0
3	H_2M_6	47.3^{A}	2.7	0.2^{B}	94.2	3.3	1.6	0.05	2.0	68.3
	H_1M_8	71.1^{A}	2.9	0.6^{B}	95.1	5.0	1.7	0.15	2.9	36.5
	S	14.3^{B}	1.7	4.1^{A}	71.1	1.0	1.0	1.0	1.0	1.0
4	H_1M_9	$27.8^{A,B}$	6.4	0.13	81.1	2.8	2.4	0.11	1.2	26.2
	H_1M_{10}	56.4^{A}	18.7	0.13	75.0	5.7	7.0	0.11	0.81	53.2
	S	9.9^{B}	2.7	1.2	71.9	1.0	1.0	1.0	1.0	1.0
5	H_1M_3	42.9^{A}	2.4^{A}	1.7^{B}	91.1	9.4	3.0	0.16	3.1	59.0
	M_3	6.7^{B}	0.20^{B}	0^{B}	97.1	1.5	0.25	—	5.8	—
	S	4.6^{B}	0.79^{B}	10.9^{A}	28.1	1.0	1.0	1.0	1.0	1.0
6	H_1M_8	32.0^{A}	2.9^{A}	0	91.8	334	28.7	—	11.1	—
	H_2M_8	41.5^{A}	1.5^{A}	0	96.5	434	15.2	—	27.3	—
	S	0.10^{B}	0.10^{B}	0	50.0	1.0	1.0	—	1.0	—
7	H_1M_9	8.1^{A}	4.2^{B}	0	65.6	5.5	1.3	—	4.3	—
	SM_9	22.7^{A}	37.6^{A}	0	37.7	15.5	11.3	—	1.4	—
	S	1.5^{B}	3.3^{B}	0	30.6	1.0	1.0	—	1.0	—

[a] Within columns and experiments, means with different superscript letters differ significantly ($P < 0.05$).

TABLE 3. BACK-TRANSFORMED MEAN DAILY FLY CATCHES AND PERFORMANCE CHARACTERISTICS FOR ATTRACTANTS BASED ON SWORMLURE-2 AND LIVER STANDARD (S)

		Mean daily catch[a]				Potency			Selectivity	
Experiment	Attractant	*L. cuprina*	*Chrysomya* spp.	*Calliphora* spp.	% *L. cuprina*	*L. cuprina*	*Chrysomya*	*Calliphora*	*L. cuprina*/ *Chrysomya*	*L. cuprina*/ *Calliphora*
8	N_1	72.9^{A}	49.0^{A}	0	59.8	6.7	245	—	0.03	—
	N_2	83.6^{A}	30.0^{A}	0	73.6	7.7	150	—	0.05	—
	S	10.8^{B}	0.20^{B}	0.31	95.5	1.0	1.0	1.0	1.0	1.0
9	N_2	7.0^{A}	28.5^{A}	1.3	19.1	16.2	66.3	4.1	0.24	4.0
	N_3	6.1^{A}	23.8^{A}	1.2	19.5	14.0	55.3	3.7	0.25	3.8
	M_1N_2	5.7^{A}	28.7^{A}	1.1	16.1	13.2	66.7	3.6	0.20	3.7
	S	0.43^{B}	0.43^{B}	0.31	37.0	1.0	1.0	1.0	1.0	1.0
10	N_2	10.7^{B}	1.2	0	90.2	64.2	3.3	—	19.7	—
	N_4	11.3^{B}	2.2	0	83.9	68.0	6.0	—	11.2	—
	H_2N_2	19.2^{A}	3.7	0	83.9	115.2	10.2	—	11.2	—
	S	0.17^{C}	0.36	0	31.8	1.0	1.0	—	1.0	—
11	H_1N_1	148.9^{A}	255.1^{A}	0.56	36.8	92.5	364	0.86	0.26	108
	H_1M_3	29.7^{B}	2.9^{B}	0.17	90.6	18.4	4.1	0.26	4.5	72.6
	S	1.6^{C}	0.72^{B}	0.66	54.0	1.0	1.0	1.0	1.0	1.0
12	H_1N_1	20.8^{A}	238.1^{A}	0^{B}	8.0	2.1	13.2	—	0.16	—
	H_2M_6	21.9^{A}	12.1^{B}	0^{B}	64.3	2.3	0.67	—	3.4	—
	S	9.7^{B}	18.1^{B}	0.80^{A}	34.0	1.0	1.0	1.0	1.0	1.0

[a]Within columns and experiments, means with different superscript letters differ significantly ($P < 0.05$).

catch, and the calculated potency and selectivity values are given for each synthetic attractant mixture and the liver/sodium sulfide standard. The mean daily catches for the different genera varied over a wide range, presumably due to changing environmental conditions and other factors such as fluctuating resources. The calculated potency values of the various attractant mixtures varied from <0.01 to >100, showing that the chemical composition of the mixtures strongly affected their relative attractancy.

The mean daily *L. cuprina* catch in the attractant/sticky trap system was above 10 flies for at least one attractant in each experiment, with the exception of experiment 9 (highest *L. cuprina* mean 7.0). The highest mean daily catches on the sticky traps were 148.9 *L. cuprina* (Exp. 11), 255 *Chrysomya* spp. (Exp. 11), and 21.1 *Calliphora* spp. (Exp. 2). Saturation of the sticky surface was estimated to require approximately 1000 flies. Thus, a trapping period of 24 hr resulted in acceptable fly numbers, prevented trap saturation, and allowed 4×4 and 5×5 Latin square experiments to be completed within 1 wk.

Liver standard generally caught fewer *L. cuprina* than synthetic attractants, with the exception of some less attractive mixtures. The highest *L. cuprina* catch with the standard was 14.3 flies (Exp. 3). At the start of this study, the liver mixture was the only standard attractant for the blowfly (Mackerras et al., 1936; Vogt and Havenstein, 1974) and, thus, the sole reference available. We limited its inherent variability (Vogt and Woodburn, 1994) by standard preparation and dispensing, use at uniform age, and use for one experiment only (4 or 5 d). The liver standard served as an indicator of the density and composition of prevailing blowfly populations and allowed us to standardize and compare results from experiments conducted in different locations, seasons, and production environments.

The effectiveness of an attractant is expressed as its potency to attract certain genera or species and as its selectivity for *L. cuprina* compared with other genera. The potency is obtained by dividing the back-transformed mean catch of the attractant by the liver standard, the potency and selectivity of which is defined as equal to 1.

Experiments 1 and 2 (Table 2) showed that the 2-me/indole/H_2S mixtures H_1M_2 and H_1M_5, the most powerful *L. cuprina* attractant to emerge from previous investigations (Eisemann, 1985), had low potency values for *L. cuprina* (<0.2). Addition of thiolacetic acid to 2-me/indole/H_2S (H_1M_4, H_1M_7) did not markedly change the attractancy for *L. cuprina*, but the inclusion of pentanoic (valeric) acid (H_1M_6, H_1M_3) boosted the catch by an order of magnitude or more. Combinations containing pentanoic acid were the only ones in experiments 1 and 2 which were more attractive to *L. cuprina* than the liver standard (potency 7.3 and 2.5, respectively). Pentanoic acid increased the catches of *Chrysomya* spp. and *Calliphora* spp. as well, but to a lesser extent than for *L. cuprina*; this resulted in a higher selectivity of these attractants for *L. cuprina* compared to other species. Substitution of pentanoic with butanoic acid (H_1M_8) provided an equally

or more potent mixture for *L. cuprina* with little change in attractancy for other blowflies (Exp. 3). The relative concentrations of 2-me and butanoic acid (M_9 4:1 v/v; M_{10} 1:4) did not significantly affect the trap catches of the three genera (Exp. 4).

The addition of butanoic or pentanoic acid to 2-me/indole/H_2S mixtures, considered to be optimal attractants in laboratory assays (Eisemann, 1985), strongly increased attraction of *L. cuprina* under field conditions. Short chain fatty acids such as acetic, butanoic, pentanoic, and hexanoic acid, have been isolated from wool wax (Freney, 1940; Nolte, 1943). They are also common metabolites of bacteria, including *P. aeruginosa*, the predominant cause of fleece rot, an important predisposing condition for fly strike (Watts et al., 1979). However, several previous studies showed no or little response or orientation of *L. cuprina* toward these short chain fatty acids alone (Freney, 1937; Hepburn and Nolte, 1943; Eisemann, 1985). In the present study, *L. cuprina* attractancy was greatly increased when these acids were combined with weakly attractive mixtures. Olfactory synergy between chemicals to provide enhanced attractancy for *L. cuprina*, as observed here, was postulated by Freney (1937).

Removal of the H_2S-producing sodium sulfide solution from a 2-me/indole/pentanoic acid/H_2S combination (M_3 vs. H_1M_3, Exp. 5) resulted in a substantial reduction in catches of all three genera. The concentration of these solutions (H_1, H_2) did not significantly alter the performance of the attractant, however (Exp. 6). This observation agreed with laboratory investigations (Urech et al., unpublished data), which have shown that the rate of H_2S release from the two solutions is similar when exposed to air. A combination of a synthetic organic attractant and a liver standard (SM_9) caught 2.5 times more *L. cuprina* than the same synthetic attractant and H_2S (H_1M_9), indicating that the liver-based mixture was emitting additional components that attract *L. cuprina*. However, the attraction of *Chrysomya* spp. to SM_9 increased by a factor of 9, thereby reducing the selectivity for *L. cuprina* against *Chrysomya* spp. from 4.3 (H_1M_9) to 1.4. This was supported by the composition of the trap catches, in which the *L. cuprina* percentage dropped from 66% using H_1M_9 to 38% using SM_9.

Swormlure-2 and some related combinations were tested for their attractancy for *L. cuprina* (Table 3). Swormlure-2 (N_1) attracted *L. cuprina* (potency 6.7) but was more effective for *Chrysomya* spp. (potency 245). A 90% reduction of dimethyl disulfide (DMDS) concentration (N_2) did not alter the *L. cuprina* catch, but the potency for *Chrysomya* spp. dropped to 150. A further 10-fold reduction of DMDS concentration (N_3) or the inclusion of 2-me (M_1N_2) only slightly altered the attraction. The addition of a sodium sulfide solution also significantly increased the *L. cuprina* catch of the N_2 attractant (Exp. 10).

Differences between attractants based on Swormlure/H_2S and those based on 2-me/indole/pentanoic acid/H_2S were compared in two trials (Table 3). In experiment 11, the Swormlure-based blend attracted significantly more *L. cuprina*

and *Chrysomya* spp. than its competitor, possibly as a result of faster evaporation of active components from N_1, because these are more volatile than those in the aqueous M_3 solution. The experiment was repeated (Exp. 12) with N_1 evaporation controlled with a wick, as used in the screwworm fly traps (Coppedge et al., 1977). The attractancy of the two mixtures for *L. cuprina* was equal, but H_1N_1 caught 20 times as many *Chrysomya* spp. as H_2M_6. This is reflected in the selectivity factor for *L. cuprina* against *Chrysomya* spp. which was 0.16 for H_1N_1 and 3.35 for H_2M_6. This difference is also seen in the composition of the trap catch, where *L. cuprina* constituted 8 and 64% of the catch for the N and M series attractants, respectively.

The sample of approximately 500 *Lucilia* spp. from trap catches in various field trials did not contain *L. sericata*. This corroborated earlier findings (Waterhouse and Paramanov, 1950) that *L. sericata* is not present in Queensland. In the cooler Canberra area (about 1000 km south of Queensland), Freney (1937) caught *L. sericata* in Western Australian blowfly traps baited with liver/sodium sulfide solution with *L. sericata* constituting 6% of the total *Lucilia* spp. catch.

Most of the *Chrysomya* spp. caught in the traps were *C. rufifacies*, with fewer *C. varipes* and *C. megacephala*/*saffranea* also present. The major *Calliphora* species was *C. augur*, and a few *C. stygia* also were trapped.

In addition to the above described field experiments, other experiments, including the same or similar attractant mixtures, were carried out in different locations under varying seasonal conditions, and with large variations in populations of blowfly genera. The attractants within the M and N series showed similar characteristics, that is, selective attractancy for *L. cuprina* or *Chrysomya* spp. The results from all experiments were pooled and used to compare 2-me/indole/acid (M) and Swormlure (N) series attractants. The results from experiments including attractants M_3, M_6, M_8–M_{10}, and N_1–N_4 were pooled. Because the data were not normally distributed, and the large range of the values, a square-root transformation of the data was used. Table 4 shows the back-transformed means for the performance of the M and N attractant series, including daily fly catches for the three genera, the percentage of *L. cuprina* in the total fly catch, the percentage of male *L. cuprina*, the potency for each genera, and the selectivity for *L. cuprina* vs. other blowfly genera.

The mean daily *L. cuprina* catches of the M and the N attractants were 5–10 times higher than with the liver standard. The mean potency values for *L. cuprina* were 19.3 and 40.1, for the M and N attractants, respectively. The higher *L. cuprina* attractancy of the N group compared to the M attractants is most likely due to increased emission of attractants because of their greater volatility. Increasing the concentration of the organic components in an M-related mixture elevates the trap catches (Urech et al., 1993). In a comparison between attractants from both groups, where the release of N was controlled with a wick (Exp. 12, Table 3), N_1 and M_6 showed the same potency for *L. cuprina*. Thus, both groups provide good

attractancy and resultant trap catches for the target species, which makes them good candidates for a synthetic *L. cuprina* attractant.

A major difference between the M and N series was their attractancy for *Chrysomya* spp., which was substantially higher for the N group. Mean daily *Chrysomya* catches were lower with the attractant than the liver standard in the M group, but about 20 times higher with the N attractants. This is seen in the much higher *Chrysomya* spp. potency for the N (57) than the M (2.74) attractants. The high potency of the N group for *Chrysomya* spp. is not surprising, as Swormlure is an attractant developed for the New World screwworm fly, *Cochliomyia hominivorax*, based on degradation products of animal protein (Jones et al., 1976). The Old World screwworm fly, *Chrysomya bezziana*, is also attracted by Swormlure (Spradbery, 1994).

The mean daily fly catches of *Calliphora* spp. were lower with the synthetic attractants than with the liver standard, particularly for the M series, which caught less than 20% of the standard catch. The potency values for *Calliphora* spp. were 0.09 and 1.06, for the M and N series, respectively. The experiments with the N attractants were carried out during summer when *Calliphora* spp. populations were lower (standard catch 0.52) than during spring and autumn when most M series trials were conducted (standard catch 3.78). In a previous study in the Canberra area, which has a cooler climate than Queensland, *Calliphora* spp. blowflies far outnumbered *L. cuprina* in all weeks from October to April (Norris, 1966).

The M series averaged a particularly high *L. cuprina* trap content (mean 84.9%), compared with 37.1% for the liver/sodium sulfide standard in the same experiments. The N group also had a higher *L. cuprina* catch (50.4%) than the standard (36.1%), but not as high as the M group, due to their higher *Chrysomya* catch. A high proportion of the target species in the trap catch is a desirable feature, bestowing several advantages. For example, in fly population monitoring traps, less sorting and separating of fly species is required, fly population suppression traps will take longer to fill up and thus need less servicing, and fewer non-target (and potentially beneficial) species are removed from the ecosystem.

Both the synthetic attractants and the liver standard attracted more female than male *L. cuprina*. The proportion of males captured was higher with the synthetic attractants than with the standard liver attractant. Carrion-based traps have shown preferential attraction for female flies (Vogt et al., 1985; Wall et al., 1992). This phenomenon was observed with synthetic attractants, but to a lesser degree. For field population monitoring, the sex ratio of the trap catch is less important. When used for population suppression, maximizing the proportion of female *L. cuprina* trapped is desirable.

The attractants of the M series were superior to those of the N series with respect to selectivity for *L. cuprina* compared to other blowflies (Table 4). The M group achieved a selectivity for *L. cuprina* of 4.69 against *Chrysomya* spp. and 36.6 against *Calliphora* spp. Thus, a trap catch of 33% each of *L. cuprina*,

TABLE 4. MEAN DAILY FLY CATCHES, % *L. cuprina*, % MALE *L. cuprina*, AND PERFORMANCE CHARACTERISTICS FOR ALL FIELD EXPERIMENTS INVOLVING ATTRACTANTS BASED ON 2-MERCAPTOETHANOL/INDOLE/BUTANOIC (PENTANOIC) ACID/HYDROGEN SULFIDE (M SERIES) OR SWORMLURE-2 (N SERIES) AND FOR A LIVER STANDARD (S) USED IN THE SAME EXPERIMENTS[a]

		M series				N series			
		Attractant	*n*	Standard	*n*	Attractant	*n*	Standard	*n*
Mean daily catches	*L. cuprina*	22.6	22	4.41	15	26.6	12	2.14	7
	Chrysomya	3.22	22	3.72	15	33.3	12	1.56	7
	Calliphora	0.65	15	3.78	10	0.43	6	0.52	4
Fly species	% *L. cuprina*	84.9	22	37.1	15	50.4	12	36.1	7
Sex	% male *L. cuprina*	19.4	22	9.83	14	20.4	12	5.28	6
Potency	*L. cuprina*	19.3	21			40.1	10		
	Chrysomya	2.74	20			57.0	12		
	Calliphora	0.09	15			1.06	6		
Selectivity	*L. cuprina*/*Chrysomya*	4.69	17			2.52	10		
	L. cuprina/*Calliphora*	36.6	9			23.3	3		

[a] Back-transformed means of square-root transformed data; n = number of experiments used for mean calculation.

Chrysomya spp., and *Calliphora* spp. with the liver standard corresponds to a catch of 81, 17, and 2%, respectively, with a synthetic attractant from the M series. Selectivity against *C. rufifacies* is particularly advantageous because their larvae are predators of *Lucilia* larvae, thus minimizing *L. cuprina* breeding in carcasses (Kitching, 1981; Cook et al., 1995). The high selectivity against *Calliphora* spp. will prove particularly useful for sheep blowfly trapping in areas with cooler conditions where *Calliphora* are more abundant (Norris, 1966). The observed selectivity for *L. cuprina* against other blowflies fulfils Mackerras' criteria of specificity for new blowfly control measures (Mackerras, 1936).

The M group attractants have additional benefits over the N group. They comprised fewer components, with boiling points (and, thus, vapor pressures) within a narrower range than the Swormlure-2 combinations (Coppedge et al., 1977). This is an advantage in the production of long-lasting, controlled-release dispensers. These baits are suitable for replacing the current standard attractant for sheep blowflies. Besides the advantage of increased *L. cuprina* catches, the synthetic attractants are not subject to variability of release, as observed with liver/sodium sulfide mixtures (Vogt and Woodburn, 1994). A synthetic attractant has a defined, standard composition and is readily packaged into a controlled-release system, emitting a constant amount of odor of known composition. These characteristics make a synthetic mixture desirable for fly population monitoring or suppression trapping in the field.

In summary, the best synthetic attractants described here are potent and selective attractants for the Australian sheep blowfly and are superior to the standard liver/sodium sulfide attractant for *L. cuprina*. The M series attractants, containing 2-me, indole, a short chain fatty acid, and a sodium sulfide solution, offer higher potency for *L. cuprina*, combined with selectivity against other blowflies. Such combinations can be packaged in a controlled-release dispenser to make convenient attractants for monitoring or suppression of *L. cuprina* populations.

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BIOACTIVITY OF PHENANTHRENES FROM *Juncus acutus* ON *Selenastrum capricornutum*

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Abstract—Twenty-five 9,10-dihydrophenanthrenes, four phenanthrenes, a dihydrodibenzoxepin, and a pyrene, isolated from the wetland plant *Juncus acutus*, were tested to detect their effects on the green alga *Selenastrum capricornutum*. Nine of the compounds were isolated and identified for the first time. Most of the compounds caused inhibition of algal growth. The 9,10-dihydrophenanthrenes **1**, **5**, **21**, and **22** were the most active.

Key Words—*Juncus acutus*, dihydrophenanthrenes, phenanthrenes, dihydrodibenzoxepin, pyrene, algiecides, *Selenastrum capricornutum*.

INTRODUCTION

As part of our effort to discover natural products with potential use as algicides, we have investigated the aerial part of *Juncus effusus* and reported that many of the 25 dihydrophenanthrenes isolated from the plant possess significant antialgal activity (DellaGreca et al., 1996, 1997). A toxicity evaluation of these compounds on aquatic species from various taxonomic groups and their possible selective toxicity in aquatic ecosystems has been also reported (DellaGreca et al., 2001a).

In continuing our studies, we have considered another Juncaceae, *Juncus acutus*, a wetland plant widely distributed in the Mediterranean area. From samples collected in Sardinia, nine 9,10-dihydrophenanthrenes, three phenanthrenes,

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and a pyrene were isolated and described in a recent paper (DellaGreca et al., 2002). Many of them have inhibitory activity on *Selenastrum capricornutum*, the algal species selected for standardized studies in aquatic environments (ISO, 1989; OECD, 1994; ASTM, 1998). The ability of some natural products to inhibit microalgae may be of ecotoxicological significance because algae are a key functional group in freshwater, and specific toxic effects may be expressed in algae at low concentrations. Furthermore, alterations of the phytoplankton community due to a toxic stress may affect the structure and functioning of the whole ecosystem (Nyholm and Källqvist, 1989).

In this paper, we report a study from samples collected in Sabaudia, near Rome. Eight new 9,10-dihydrophenanthrenes and a phenanthrene were isolated in addition to already reported compounds that were identified on the basis of their spectroscopic features.

METHODS AND MATERIALS

Plant Material. *Juncus acutus* plants were collected on land 300 m from the sea, in Sabaudia (near Rome) during summer (July). They were identified by Professor Antonino Pollio of the Dipartimento di Biologia Vegetale of University of Naples Federico II. A voucher specimen (HERBNAPY250) was deposited at the Dipartimento di Biologia Vegetale of University of Naples Federico II.

General Experimental Procedures. NMR spectra were recorded at 500 MHz for ^{1}H and 125 Hz for ^{13}C on a Varian Unity Inova spectrometer in $CDCl_3$ or CD_3OD solutions, at 25°C. Proton-detected heteronuclear correlations were measured using HMQC (optimized for $^{1}J_{HC} = 160$ Hz) and HMBC (optimized for $^{1}J_{HC} = 7$ Hz). IR spectra were determined in $CHCl_3$ solutions on a FT-IR Perkin-Elmer 1740 spectrometer. UV spectra were obtained on a Perkin-Elmer Lambda 7 spectrophotometer in $CHCl_3$ solutions. MS spectra were obtained with an HP 6890 spectrometer equipped with a MS 5973 N detector. The HPLC was performed on an Agilent 1100 series apparatus by using a UV detector. Preparative HPLC was performed by using RP-8 (Luna 10 μm, 250 $\times$ 10 mm ID, Phenomenex), SiO_2 (Maxsil 10 μm, 250 $\times$ 10 mm ID, Phenomenex), or RP-18 (Kromasil 10 μm, 250 $\times$ 10 mm ID, Phenomenex) columns. Analytical TLC was performed on Merck Kieselgel 60 F_{254} or RP-18 F_{254} plates with 0.2 mm layer thickness. Spots were visualized by UV light or by spraying with H_2SO_4–AcOH–H_2O (1:20:4). The plates were then heated for 5 min at 110°C. Preparative TLC was performed on Merck Kieselgel 60 F_{254} plates, with 0.5 or 1 mm film thickness. Flash column chromatography (FCC) was performed on Merck Kieselgel 60 (230–400 mesh) at medium pressure. Column chromatography (CC) was performed on Merck Kieselgel 60 (70–240 mesh).

Extraction and Isolation of 9,10-Dihydrophenanthrenes and Phenanthrenes from J. acutus. Air-dried plants (6 kg) were sequentially and exhaustively extracted

with light petrol (20 l), ethyl acetate (EtOAc, 18 l), and methanol (MeOH, 18 l) at room temperature for 10 d. The light petrol extract (130 g) was dissolved in ethyl ether (900 ml) and shaken with 2 M NaOH (2 × 400 ml). The organic layer was washed until neutral and dried on Na_2SO_4 to give, after evaporation of solvent in vacuo, 65 g of neutral material. The alkaline solutions were reacidified with 2 M HCl and extracted with EtOAc. The organic layer was washed until neutral and dried on Na_2SO_4 to give, after evaporation of solvent in vacuo, 40 g of acidic material.

Neutral Fraction Separation. The neutral fraction (65 g) was chromatographed on silica gel (400 g). The fractions eluted with hexane were separated by silica gel flash column chromatography (9:1 hexane–Et_2O) to give pure **1** (21 mg), **4** (24 mg), and **17** (30 mg). Fractions eluted with hexane–$CHCl_3$ (4:1) were purified by preparative TLC (3:1 hexane–Et_2O) to give pure **25** (8 mg) and **26** (10 mg). Fractions eluted with hexane–$CHCl_3$ (3:1) were purified on an HPLC silica gel column (9:1 hexane–acetone) to give pure **6** (25 mg) and **8** (7 mg). Fractions eluted with $CHCl_3$ (100%) were separated by flash column chromatography (9:1 $CHCl_3$–acetone) and successively purified by preparative TLC (9:1 $CHCl_3$–EtOAc) to give pure **5** (16 mg), **15** (18 mg), **20** (12 mg), and **21** (15 mg).

The EtOAc extract (200 g) was separated into an acidic (60 g) and a neutral fraction (80 g), as previously described for the light petrol extract.

Neutral Fraction Separation. The neutral fraction was chromatographed on silica gel (300 g) by using a hexane EtOAc gradient, and the fractions eluted with hexane–EtOAc (9:1) were further separated by flash column chromatography (9:1 hexane–EtOAc) to give 10 fractions. Subfractions 2–5 were purified by HPLC silica gel column (4:1 hexane–EtOAc) to give pure **22** (7 mg), **28** (23 mg), and **29** (15 mg). Subfractions 6–10 were purified by HPLC silica gel column (9:1 hexane–EtOAc) to give pure **2** (5 mg) and **3** (8 mg). The acidic fraction was separated by flash column chromatography by using a $CHCl_3$–EtOAc gradient. Fractions eluted with $CHCl_3$–EtOAc (6:1) were further purified by HPLC C-18 column (2:2:1 MeOH–H_2O–MeCN) to give pure **27** (45 mg) and **31** (18 mg). Fractions eluted with $CHCl_3$–EtOAc (1:1) were rechromatographed on silica gel (24:1 $CHCl_3$–MeOH) to give eight fractions. The fractions 1 and 2 were purified by HPLC C-18 column (5:4:1 MeOH–H_2O–MeCN) to give pure **7** (15 mg), **16** (18 mg), and **19** (4 mg). Fraction 3 was purified by preparative TLC (3:1 hexane–EtOAc) to give pure **18** (9 mg). Fractions 4–6 were purified by HPLC NH_2 column (49:1 $CHCl_3$–MeCN) to give **10** (6 mg), **11** (4 mg), and **30** (9 mg). Fractions 7 and 8 were purified by HPLC C-18 column (5:3:2 MeOH–H_2O–MeCN) to give pure **12** (5 mg), **13** (6 mg), and **14** (4 mg). Fractions eluted with EtOAc were rechromatographed on silica gel (17:3 $CHCl_3$–acetone 3) and purified by HPLC C-18 column (6:3:1 MeOH–H_2O–MeCN) to give pure **9** (10 mg), **23** (8 mg), and **24** (11 mg).

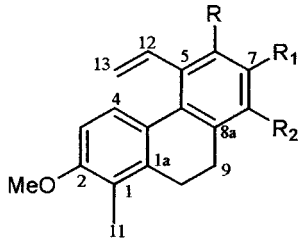
Toxicity Testing. The bioactivity of all the compounds was tested on the freshwater green alga *Selenastrum capricornutum*. The 9,10-dihydrophenanthrenes

(**1**, **4**–**7**, **10**–**13**, **15**–**17**, **20**–**22**) were previously assayed using the algal growth inhibition method in a 4-d static test with laboratory cultures of algae (DellaGreca et al., 1996, 1997). The algal growth inhibition tests for the compounds isolated only from *J. acutus* were run in 72 hr according to the International Standard Organization procedure 8692 (ISO, 1989). The algal inoculum was taken from an exponentially growing preculture and added to 25 ml of test solution to obtain an initial cell density on the order of 10^4 cells/ml. Each compound was tested in five concentrations, with three replicates per concentration and a negative control. All chemicals were dissolved in dimethyl sulfoxide (DMSO), and in the final test solutions DMSO concentration was kept constant at 0.01% (v/v). A solvent only control was included in each experiment to detect the possible effect of the DMSO. Flasks were placed in a growth chamber at 25°C under continuous illumination (8000 lux). Cell density was measured at 0 time and every 24 hr for 3 d by an electronic particle dual threshold counter (Coulter Counter Z2, 100 μm capillary, Instrumentation Laboratory, Miami, FL, USA), and from these data the algal growth inhibition was calculated by integrating the mean values from t_0 to t_{72} hr (area under the curve). Inhibition (percentage) values were reported against log-transformed data of concentrations (in μM) and processed by a regression analysis technique to obtain the respective IC_{50} value (the test concentration corresponding to 50% reduction in growth relative to the control).

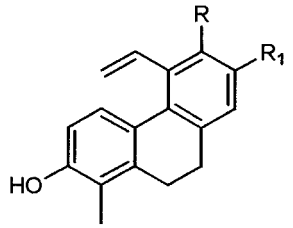
IC_{50} values were also calculated from the results of previous studies (DellaGreca et al., 1996, 1997) to allow a comparison of algal growth inhibition for all compounds isolated from *J. acutus*.

RESULTS AND DISCUSSION

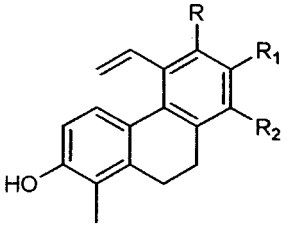
The whole plants (aerial and rhizome) were extracted with solvents of increasing polarity. Preliminary assays on *S. capricornutum* showed an algal growth inhibition of petrol (50%, 1.0 mg/l) and ethyl acetate extracts (60%, 1.0 mg/l), while the MeOH extract was inactive. The examination of the active extracts of *J. acutus* revealed 9,10-dihydrophenanthrenes, phenanthrenes, a dihydrodibenzoxepin, and a pyrene (Figures 1 and 2). Some of them had been previously isolated from another Juncaceae, *J. effusus*, and were identified by comparison of NMR data for the compounds **1**, **4**–**7**, **10**–**13**, **15**–**17**, **19**–**22** with NMR data from the literature (DellaGreca et al., 1993a,b, 1996, 1997). The examination of petrol and ethyl acetate extracts of *J. acutus* collected in Sabaudia revealed known 9,10-dihydrophenanthrenes **25** and **26**, phenanthrenes **27**–**29**, and pyrene **31** (DellaGreca et al., 2002) together with the novel compounds **2**, **3**, **8**, **9**, **14**, **18**, **23**, **24**, and **30**. Compound **2** had in the EIMS spectrum a molecular ion peak at *m*/*z* 266 and showed 18 carbon signals in the ^{13}C NMR spectrum (Table 2), consistent with a molecular formula of $C_{18}H_{18}O_2$. A DEPT experiment defined the carbons as two methyls, three methylenes, five methines, and eight quaternary carbons.



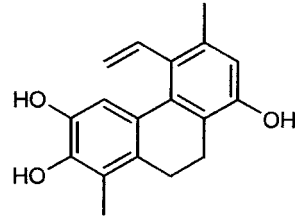
1 R = Me R_1 = H R_2 = OH
2 R = H R_1 = OH R_2 = H
3 R = H R_1 = Me R_2 = H



4 R = H R_1 = Me
5 R = Me R_1 = OH
6 R = OH R_1 = Me
7 R = H R_1 = OH
8 R = Me R_1 = H
9 R = CH_2OH R_1 = OH
10 R = H R_1 = CH_2OH
11 R = CH_2OH R_1 = H



12 R = H R_1 = H R_2 = COOH
13 R = H R_1 = COOH R_2 = H
14 R = COOH R_1 = H R_2 = H
15 R = H R_1 = OH R_2 = Me
16 R = Me R_1 = H R_2 = OH
17 R = H R_1 = OMe R_2 = Me



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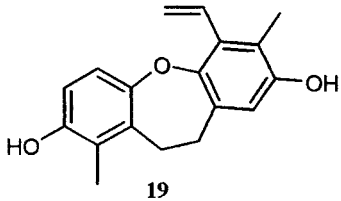

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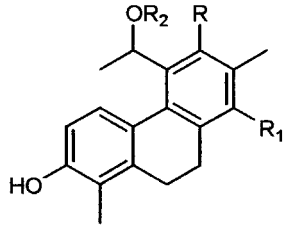
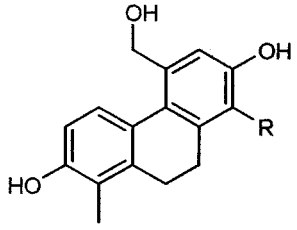
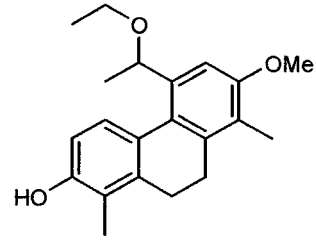


20 R = H R_1 = OH R_2 = H
21 R = OH R_1 = H R_2 = H
22 R = OH R_1 = H R_2 = Me

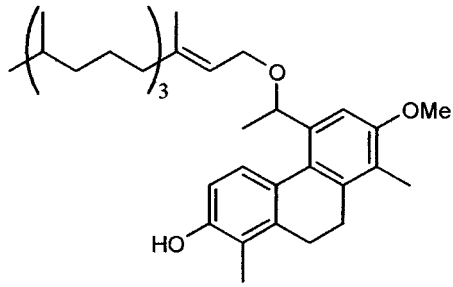
FIG. 1. Structures of 9, 10-dihydrophenanthrenes.



23 R = H
24 R = Me



25



26

FIG. 1. CONTINUED.

The ^{1}H NMR spectrum (Table 1) showed four aromatic protons, three vinyl protons, a methoxyl, two methylenes, and a methyl. These data resembled those of effusol (**7**), except for the presence of a methoxyl group. In a NOE experiment, the methoxyl had relation with the doublet at δ 6.78 and was assigned to the H-3 proton.

The EIMS spectrum of compound **3** had a molecular ion peak at m/z 264 and showed 19 carbon signals in the ^{13}C NMR spectrum (Table 2), consistent with a molecular formula of $C_{19}H_{20}O$. A DEPT experiment defined the carbons as three methyls, three methylenes, five methines, and eight quaternary carbons. The ^{1}H NMR spectrum (Table 1) showed four aromatic protons, three vinyl protons, a methoxyl, two methylenes, and two methyl groups. These data resembled those of juncunol (**4**), except for the presence of a methoxyl group. In a NOE experiment, the methoxyl had relation to the doublet at δ 6.73 and was assigned to the H-3 proton. Compound **8** had molecular formula $C_{18}H_{18}O$ according to the molecular ion at m/z 250 in the EIMS spectrum. Four aromatic *ortho*-coupled protons, three double doublets of a vinyl group, two methylene multiplets, and two methyl singlets were present in the ^{1}H NMR spectrum (Table 1). Thirteen carbon signals were present in the ^{13}C NMR spectrum (Table 2), identified by DEPT experiment as

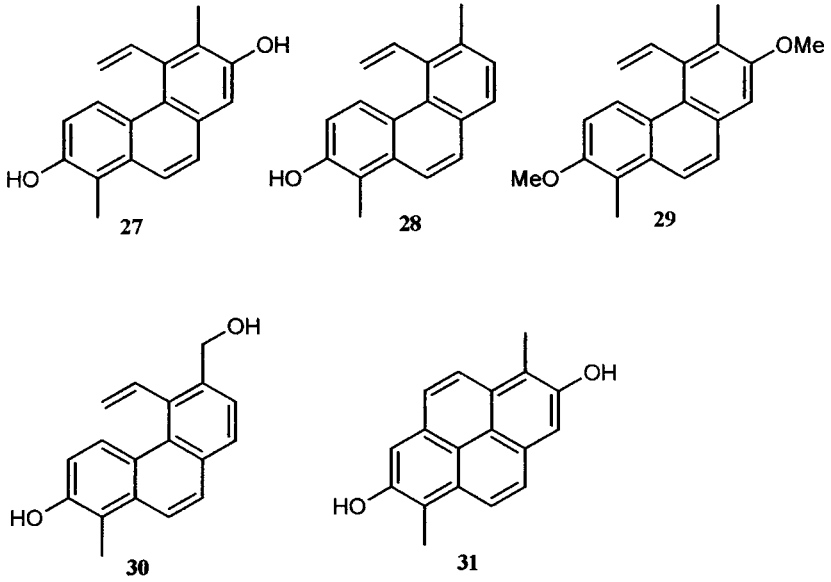


FIG. 2. Structures of phenanthrenes and pyrene.

two methyls, three methylenes, five methines, and eight quaternary carbons. The protons were assigned to the corresponding carbons by an HMQC experiment, and in combination with an HMBC experiment (Figure 3), allowed assignment of the structure 2-hydroxy-1,6-dimethyl-5-vinyl-9,10-dihydrophenathrene to compound **8**. In the HMBC spectrum, correlations between the H-12 and H-14 protons with the C-5 and C-6 carbons were observed. Furthermore, the H-7 and H-8 protons were correlated to the C-6 and C-8a carbons. The assignment of the methyl at C-6 was also confirmed by NOE between that methyl and the H-7 and H-12 protons.

The molecular ion at *m/z* 282 in the EIMS spectrum and the presence of 18 signals in the ^{13}C NMR spectrum defined a molecular formula $C_{18}H_{18}O_3$ for compound **9**. A DEPT experiment defined the carbons as a methyl, four methylenes, four methines, and nine quaternary carbons. The ^{1}H NMR spectrum (Table 1) showed the H-3 and H-4 doublets, the H-8 singlet, the H-9 and H-10 broad singlet, the H-12 and H-13 double doublets, and the H-11 and H-14 singlets. The protons were assigned to the corresponding carbons by an HMQC experiment that, in combination with an HMBC experiment, allowed the assignment of the structure 2,7-dihydroxy-6-hydroxymethyl-1-methyl-5-vinyl-9,10-dihydrophenathrene to the compound. In the HMBC spectrum, correlations between the H-12 and hydroxymethyl protons with the C-5 and C-6 carbons were observed. Furthermore, the H-8 proton was correlated to the C-6, C-7, and C-8a carbons. In addition, a NOE correlation between the hydroxymethyl protons and the H-12 proton was

TABLE 1. ^{1}H NMR DATA FOR **2**, **3**, **8**, **9**, **14**, **18**, **23**, **24**, **30** ($CDCl_3$)[a]

Position	**2**	**3**	**8**	**9**	$\Delta 4$[b]	**18**	**23**[b]	**24**[b]	**30**[b]
3	6.78 d (8.2)	6.78 d (8.6)	6.73 d (8.4)	6.77 d (8.4)	6.85 d (8.4)		6.68 d (8.0)	6.67 d (8.0)	7.20 d (9.0)
4	7.40 d (8.2)	7.48 d (8.6)	7.47 d (8.4)	7.65 d (8.4)	7.54 d (8.4)	7.44 s 7.44 s	7.33 d (8.0)	7.28 d (8.0)	8.45 d (9.0)
6	6.88 d (2.5)	7.25 s					6.90 d (2.5)	6.90 s	
7			7.12 d (7.8)		7.65 d (8.3)	6.68 s			8.56 d (8.5)
8	6.69 d (2.5)	7.02 s	7.49 d (7.8)	6.97 s	7.76 d (8.3)		6.64 d (2.5)		7.72 d (8.5)
9	2.72 m	2.74 m	2.89 m	2.66 brs	2.88 m	2.71 m	2.62 brs	2.63 brs	8.04 d (9.5)
10	2.72 m	2.74 m	2.78 m	2.66 brs	2.74 m	2.64 m	2.62 brs	2.63 brs	7.89 d (9.5)
11	2.24 s	2.26 s	2.24 s	2.29 s	2.19 s	2.32 s	2.19 s	2.18 s	2.54 s
12	6.93 dd (17.4, 11.2)	6.99 dd (18.0, 10.5)	6.77 dd (17.8, 11.6)	6.70 dd (18.4, 11.4)	7.11 dd (18.0, 11.2)	6.86 dd (17.6, 11.8)	4.66 s 4.66 s	4.64 s 4.64 s	7.18 dd (17.5, 11.5)
13	5.68 dd (17.4, 1.8), 5.26 dd (11.2, 1.8)	5.71 dd (18.0, 1.5), 5.25 dd (10.5, 1.5)	5.60 dd (11.6, 2.0), 5.22 dd (17.8, 2.0)	5.77 dd (11.4, 1.8), 5.70 dd (18.4, 1.8)	5.46 dd (11.2, 1.8), 5.19 dd (18.0, 1.8)	5.46 dd (11.8, 2.1), 5.14 dd (17.6, 2.1)			5.84 dd (11.5, 2.0), 5.45 dd (17.5, 2.0)
14		2.38 s	2.32 s	5.01 s		2.23 s		2.21 s	4.90 s
OMe	3.86 s	3.87 s							

[a] *J* values (in Hz) in parentheses.
[b] Recorded in CD_3OD.

TABLE 2. ^{13}C NMR DATA FOR **2**, **3**, **8**, **9**, **14**, **18**, **23**, **24**, **30** ($CDCl_3$)

Position	**2**	**3**	**8**	**9**	**14**[a]	**18**	**23**[a]	**24**[a]	**30**[a]
1	120.3	122.8	120.7	122.4	123.6	122.0	122.4	121.9	116.7
2	155.8	156.6	153.7	155.6	153.1	150.3	155.5	155.2	152.4
3	107.4	107.0	113.8	113.1	110.3	140.2	113.1	112.9	115.7
4	128.0	127.5	128.8	127.6	124.5	113.2	127.7	127.6	121.7
5	136.2	135.0	135.0	142.1	134.4	137.9	139.6	136.1	134.5
6	112.5	127.1	135.6	127.6	129.3	137.5	115.1	115.1	134.1
7	154.6	135.7	123.0	156.8	119.3	129.2	156.5	154.8	125.5
8	114.0	127.7	123.0	115.1	120.3	152.4	116.7	121.6	123.2
9	30.0	29.9	26.8	28.3	23.5	30.3	32.3	27.7	120.4
10	26.1	25.6	26.0	27.1	23.5	25.9	27.2	26.9	120.5
1a	139.2	139.4	135.6	154.9	135.7	129.2	153.8	140.2	131.3
4a	127.0	126.6	129.0	119.2	129.3	127.7	111.3	129.1	123.4
5a	127.4	131.3	134.7	123.2	131.8	137.9	127.0	128.1	127.4
8a	139.3	138.8	129.0	136.7	135.3	120.8	129.6	140.2	129.7
11	11.8	11.7	12.1	12.0	7.8	12.4	12.3	12.1	9.8
12	138.0	139.0	128.8	139.7	133.2	137.9	64.6	64.5	132.9
13	113.5	113.6	113.8	116.7	115.6	119.8			120.7
14		21.1	21.3	62.7	168.0	13.2			61.8
OMe	55.5	55.8							

[a] Recorded in CD_3OD.

observed. The molecular ion at *m*/*z* 280 in the EIMS spectrum and the presence of 18 signals in the ^{13}C NMR spectrum (Table 2) defined a molecular formula of $C_{18}H_{16}O_3$ for compound **14**. Its IR spectrum showed the presence of hydroxyl and carboxyl functions with absorptions at 3340, 3200, and 1682 cm^{-1}. A DEPT experiment defined the carbons as a methyl, three methylenes, five methines, and nine quaternary carbons. Four aromatic *ortho*-coupled protons, three double doublets of a vinyl group, two methylene multiplets, and a methyl singlet were present in the ^{1}H NMR spectrum (Table 1). These data were similar to those of compound **12** previously isolated from *J. effusus* (DellaGreca et al., 1993b). However, the different correlations observed in the HMBC spectrum defined the isomeric structure with the carboxyl group at C-6. The H-12 vinyl and H-7 protons gave correlations with the C-5 and C-6 quaternary carbons. Confirmation of this was given by the NOE interaction observed between the methoxyl, of the methylated **14**, and the H-12 and H-7 protons.

The structure of 2,3,8-trihydroxy-1,6-dimethyl-5-vinyl-9,10-dihydrophenanhrene was attributed to compound **18**. It had the molecular formula $C_{18}H_{18}O_3$ with the molecular ion at *m*/*z* 282 in the EIMS spectrum. The ^{1}H NMR spectrum (Table 1) showed the H-4 and H-7 singlets, the H-9 and H-10 multiplets, the H-12 and H-13 double doublets, the H-11 and H-14 singlets. In the HMBC spectrum, the H-4 proton was correlated to the C-2, C-3, C-1a, C-4a, and C-5a carbons. The

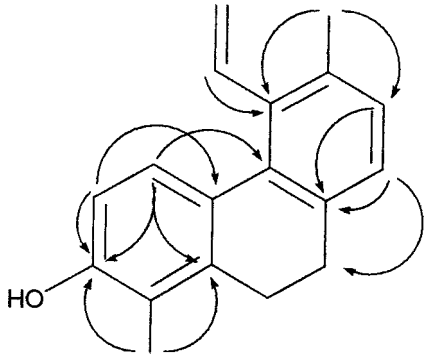


FIG. 3. Selected HMBC correlations for 9,10-dihydrophenanthrene **8**.

H-7, H-12 vinyl, and H-14 methyl gave cross peaks with the C-5, C-6 carbons and the first proton was also correlated to C-8 and C-8a. As expected from the structure, a NOE interaction between the H-14 methyl and H-7 proton was seen.

Compound **23** had the molecular formula $C_{16}H_{16}O_3$ with the molecular ion at *m*/*z* 256 in the EIMS spectrum. Accordingly, 16 carbon signals were present in the ^{13}C NMR spectrum (Table 2), identified by a DEPT experiment as a methyl, three methylenes, four methines, and eight quaternary carbons. Four aromatic protons (two *ortho*- and two *meta*-coupled), three methylenes (one singlet and two multiplets), and a methyl singlet were present in the ^1H NMR spectrum (Table 1). The protons were assigned to the corresponding carbons by an HMQC experiment and, in combination with an HMBC experiment, the structure 2,7-dihydroxy-5-hydroxymethyl-1-methyl-9,10-dihydrophenathrene was assigned to compound **23**. In the HMBC spectrum, the H-6 and H-12 protons showed correlations with the C-5 and C-5a carbons. The first proton was also correlated to the C-7 and C-8 carbons. The assignment of the hydroxymethyl at C-5 was also confirmed by the NOE between these protons and the H-6 proton.

The molecular ion at *m*/*z* 270 in the EIMS spectrum and the presence of 17 signals in the ^{13}C NMR spectrum defined a molecular formula $C_{17}H_{18}O_3$ for compound **24**. A DEPT experiment defined the carbons as two methyls, three methylenes, three methines, and nine quaternary carbons. The ^1H NMR spectrum (Table 1) showed the H-3 and H-4 doublets, the H-6 singlet, the H-9 and H-10 broad singlet, the H-12 singlet, and the H-11 and H-14 methyl singlets. The protons were assigned to the corresponding carbons by an HMQC experiment that, in combination with an HMBC experiment, allowed a structure of 2,7-dihydroxy-5-hydroxymethyl-1,8-dimethyl-9,10-dihydrophenathrene to be assigned to compound **24**. In the HMBC spectrum, correlations of the H-12 and H-6 protons with the C-5 and C-5a carbons were observed. Furthermore, the H-6

proton was correlated to the C-7 and C-8 carbons. A NOE correlation between the hydroxymethyl and the H-6 protons was observed.

Structure 2-hydroxy-6-hydroxymethyl-1-methyl-5-vinyl-phenanthrene was attributed to compound **30**. The molecular peak at *m/z* 264 in the EIMS spectrum defined the molecular formula $C_{18}H_{16}O_2$. The ^{1}H NMR (Table 1) exhibited six aromatic protons *ortho*-coupled, three vinyl protons as double doublets, and a methylene and a methyl singlet. In the ^{13}C NMR spectrum (Table 2), 18 carbon signals were present, which were defined by a DEPT experiment as a methyl, two methylenes, seven methines, and eight quaternary carbons. The HMBC spectrum showed cross peaks of the H-11 methyl protons with the C-1, C-2, and C-1a carbons. The H-3 proton was heterocorrelated with the C-1, C-2, and C-4a carbons. According to the ^{1}H-^{1}H COSY, the proton at δ 8.45 was attributed to the H-4. It gave interactions with the C-2, C-1a, and C-5a carbons. The interaction of the H-12 vinyl proton to the C-5a carbon located the vinyl chain at C-5 and, accordingly, this carbon was correlated with the H-13 protons. In the same experiment, both the H-7, at δ 8.56, and the H-14 methylene protons were correlated to the C-5 carbon according to the location of the hydroxymethyl at the C-6 position. Finally, the H-9 proton gave cross peaks with the C-1a and C-5a, and the H-10 proton gave cross peaks with the C-1 and C-4a carbons. A NOE correlation between the H-14 hydroxymethyl and the H-7 proton was observed.

The phytoxicity of all the compounds was tested on the freshwater green alga *Selenastrum capricornutum* and the respective median inhibition concentrations (IC_{50}) are reported in Table 3. Among the substances tested, the most active was compound **1** ($IC_{50} = 11.1\ \mu$M) with an activity of 9, and 11 times that of compounds **3** (103.4 μM) and **31** (126.8 μM), respectively, that showed the least toxic values in IC_{50}. However, compounds **7**, **8**, **18**, **19**, **23**, and **30** did not reach an IC_{50} value, showing only slight inhibition at the highest concentrations assayed. Two compounds, **26** and **29**, were found to biostimulate the algal growth even at higher concentrations (90% at concentration of 410.9 μM for **29**).

The presence of one hydroxyl on the molecule seems important for the activity. Compound **4** is twice as active as compound **3**, which does not have a hydroxyl group. Compounds **1**, **4**, and **17**, which have a single hydroxyl group, are six, three, and two times, respectively, more toxic than **15**, **7**, and **16** that have two hydroxyl groups. For compound **18**, the presence of three hydroxyl groups cause a further reduction of the activity (35% inhibition at 82.1 μM). The isomeric compounds **5** and **6**, **10** and **11**, **12**, **13**, and **14** exhibit comparable responses, indicating that the relative position of the groups is not very important. The presence of a vinyl, a hydroxyethyl, or a hydroxymethyl at C-5 does not cause significant differences in the antialgal activity; compare the isomeric **6**, **7**, and **15** with **21**, **22**, **23**, and **24**. The presence of a phytoxyl group in compound **26** causes a biostimulation of algal growth. Phenanthrenes **27** and **30** are less active than the corresponding

TABLE 3. MEDIAN INHIBITION CONCENTRATIONS (IC_{50}) FOR *Selenastrum capricornutum*

Compound	IC_{50} (μM)	95% confidence interval
1	11.1	8.6–15.3
2	23.8	21.2–26.7
3	103.4	92.2–116.1
4	45.6	37.6–58.0
5	19.9	12.8–35.7
6	26.2	20.3–33.7
7	15% inhibition at 100.0 μM	
8	36% inhibition at 120.0 μM	
9	65.0	55.7–75.5
10	77.5	59.6–100.8
11	66.4	35.2–125.3
12	26.0	21.8–31.0
13	27.0	22.4–32.6
14	54.1	47.6–62.6
15	69.0	43.6–185.6
16	68.0	61.4–76.0
17	28.0	24.5–32.4
18	35% inhibition at 82.1 μM	
19	19% inhibition at 106.4 μM	
20	35.6	29.9–41.6
21	16.8	7.5–25.5
22	16.2	13.9–18.9
23	7% inhibition at 89.8 μM	
24	75.3	56.4–100.4
25	49.7	43.2–58.3
26	30% biostimulation at 43.4 μM	
27	72.9	67.9–78.4
28	83.1	63.9–119.1
29	90% biostimulation at 410.9 μM	
30	27% inhibition at 125.0 μM	
31	126.8	114.2–139.5

9,10-dihydrophenanthrenes **5** and **11**. On the contrary, phenanthrene **28** is more active than 9,10-dihydrophenanthrene **8**. Structure–activity studies carried out on synthetic phenanthrenes and dihydrophenanthrenes also indicated that the first compunds were less active than the second (DellaGreca et al., 2000, 2001a,b). The phytotoxicity of phenanthrenes components might justify the reported chemical interaction toward dominance of *Juncus* over species from Cyperaceae found growing sympatric with, but subordinate to, *Juncus* and the autotoxicity response in these species (Ervin and Wetzel, 2000).

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Book Review

Pheromones and Animal Behaviour: Communication by Smell and Taste. By Tristram D. Wyatt. Cambridge University Press, Cambridge, UK. $100 (Hardback), $40 (Paperback). ISBN 052148068X

Over the past 10 years or so, there has been an explosion in research in the chemical senses that has been fueled largely by advances in molecular biology. For example, large families of genes controlling the expression of putative olfactory receptors have been identified in vertebrates and invertebrates alike. What are the ligands, however, that bind to these receptors? Some of them will surely be chemicals that are used to communicate between individuals of the same species: pheromones. This book by Tristram Wyatt examines the role of pheromones in the lives of animals through the looking glass of behavior. It is refreshing to see an all-inclusive approach to this topic, one that celebrates the diversity of animal life rather than reducing it to a couple of token "model" systems. It has been a long time since anyone burdened themselves with such a broad undertaking, so this book should be well received by ethologists and chemical ecologists alike. Of course, cutting a swath across invertebrate and vertebrate taxa could have produced a substantial volume. That compulsion has been resisted here to produce a well-written book that provides enough details to be convincing without devoting too much page space to any one specific subject. The overall shape of the book is aided by frequent references to both the primary literature and more in-depth specific review articles. In addition, many points are supported by use of data and illustrations from these sources. As such, the book will be particularly accessible to undergraduates and educators. More specialized practitioners of chemical ecology and behavior will also find something new and interesting in this broad text, and a gateway to original articles for those seeking a more detailed treatment is available through the cited source material. Many books currently are a compilation of chapters written by various experts in their fields. Such tomes often make for choppy reading owing to the different styles, both in prose and theme, of the contributing authors. Another strength of this volume is that it has been penned by a single author, allowing the development of consistent themes in each chapter that revolve, of course, around pheromones and behavior, but that are also placed within a broader evolutionary framework. Wyatt resists the temptation to pedal his own opinions, although there are times when some ideas could use further development rather than compression into a single-sentence question.

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Although the book is written with great clarity throughout, there were several chapters that I found particularly noteworthy. One entitled "Sex pheromones: Finding and choosing mates" offered a concise introduction to mate choice and sexual selection, and another "Pheromones and social organization" provided details of how pheromones are used by certain animal groups to maintain a coherent social structure. A contrasting set of social interactions, that of establishing and maintaining territory, were examined separately in "Scent marking and territorial behaviour." Recent advances in our understanding of how chemical information is detected, processed, and represented by the brain were discussed in "Perception and action of pheromones: From receptor molecules to brains and behaviour." "Finding the source: Pheromones and orientation behaviour" explained the behavioral mechanisms that animals employ to navigate along pheromone plumes and trails. An additional chapter "Breaking the code: Illicit signallers and receivers of semiochemical signals" on those animals capable of varying degrees of chemical espionage and subterfuge was especially fascinating. A final one "On the scent of human attraction: Human pheromones" discussed the importance of olfactory information to humans and presented evidence for the existence of human pheromones.

All in all this is an excellent general book at a reasonable price. Color figures could have been used in certain instances, but that would likely have increased the cost. Appropriately, the book is dedicated in part to Martin Birch who was an early pioneer in insect pheromone research and edited one of the first, and possibly last, book devoted to the general subject of pheromones (Birch, 1974). There have been other recent volumes (Cardé and Minks, 1997; Hardie and Minks, 1999; Vander Meer et al., 1998), but they have tended to be aimed at a narrower, more specialized audience and all focus on insect pheromone systems. Although a great deal of progress has been made since Birch's compilation both in terms of identifying the actual chemicals that constitute pheromones and the behaviors that they elicit, it is clear from Wyatt's treatise that an enormous amount of work remains to be done. Phero-men, women, and students should be busy for many years to come!

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COLORADO POTATO BEETLE TOXINS REVISITED: EVIDENCE THE BEETLE DOES NOT SEQUESTER HOST PLANT GLYCOALKALOIDS

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Abstract—The Colorado potato beetle feeds only on glycoalkaloid-laden solanaceous plants, appears to be toxic to predators, and has aposematic coloration, suggesting the beetle may sequester alkaloids from its host plants. This study tested 4th instars and adults, as well as isolated hemolymph and excrement, to determine if the beetles sequester, metabolize, or excrete alkaloids ingested from their host plants. HPLC analysis showed: that neither the larvae nor the adults sequestered either solanine or chaconine from potato foliage; that any alkaloids in the beetles were at concentrations well below 1 ppm; and that alkaloids were found in the excrement of larvae at approximately the same concentrations as in foliage. Analysis of alkaloids in the remains of fed-upon leaflet halves plus excreta during 24 hr feeding by 4th instars, as compared to alkaloids in the uneaten halves of the leaflets, showed that equal amounts of alkaloids were excreted as were ingested. The aposematic coloration probably warns of a previously-identified toxic dipeptide instead of a plant-derived alkaloid, as the Colorado potato beetle appears to excrete, rather than sequester or metabolize, the alkaloids from its host plants.

Key Words—*Leptinotarsa decemlineata*, Chrysomelidae, sequestration, alkaloid, aposematic, leptinotarsin, specialist herbivore, solanaceous, olanine, chaconine.

INTRODUCTION

The Colorado potato beetle (CPB) (*Leptinotarsa decemlineata*) (Coleoptera: Chrysomelidae) feeds exclusively on solanaceous plants in both the larval and adult stages. Solanaceous plants contain glycoalkaloids, a family of steroidal compounds that can disrupt cellular membranes and act as acetylcholinesterase

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inhibitors (Friedman and McDonald, 1997). The beetle has conspicuous coloring as an orange larva with black spots around the spiracles, and as a yellow-orange adult with black stripes on the elytra. Both intact beetles and CPB hemolymph are repellant to ants (Deroe and Pasteels, 1977), and chickens learn to avoid feeding on the distasteful larvae and adults (Hough-Goldstein et al., 1993). The repellency to predators, aposematic coloration, and feeding on solanaceous plants suggest that the beetle sequesters glycoalkaloids from the foliage of its host plants, as do many aposematic insects that are specialist herbivores on a group of highly toxic plants (Rothschild, 1972). A variety of other chrysomelid beetles sequester toxic compounds from host plants (Pasteels et al., 1990, 2001), presumably as a relatively energetically inexpensive protection against natural enemies.

The experiments described examine both the CPB and its excrement for evidence of glycoalkaloid sequestration or excretion by the beetle. Other compounds that can protect the CPB from potential natural enemies are briefly discussed. The connection between conspicuous coloration, plant secondary compounds, and specialist feeders is reviewed.

METHODS AND MATERIALS

Field-collected Beetles. Beetles to be assayed for glycoalkaloid content were collected as late 4th instars in potato (*Solanum tuberosum* L., cultivar Russet Burbank) fields in Hermiston, Oregon, USA, in August 2000 and July and August 2001. Beetles were frozen live at −20°C either as 4th instars or as adults after feeding *ad libitum* on potato plant cuttings and then pupating in sterile moistened sand in the laboratory. Excrement from 4th instars was collected in a tap-water rinse of the leaves and the plastic bucket in which the beetles were caged while feeding, and excess water was allowed to sublimate in the −20°C freezer. Additionally, hemolymph was collected with a capillary tube pressed against a small slit cut in the abdomen of each of 15 live ethanol-surface-sterilized 4th instars, pooled, and frozen for analysis. All samples were dried 2–3 d at 45°C prior to grinding with a mortar and pestle for extraction. A total of 7 sets of 4th instars (5 individuals/set) and 4 sets of adults (6 adults/set), as well as one batch of hemolymph and one of excrement, were dried and ground for extractions.

Laboratory Assay. In order to determine if 4th-stage beetles excrete all the glycoalkaloids they ingest, they were tested in a split-leaf assay. The assays utilized beetles reared for 3–5 generations in a laboratory colony containing beetles from Oregon and Michigan, and they were fed *ad libitum* on whole growth-chamber-grown potato plants (cultivar Russet Burbank). Both the beetles and plants were held at 16:8 L:D at approximately 35°:25°C in a chamber with 400 watt high-pressure sodium lamps with plant-grow broad-spectrum fluorescent bulbs providing the lighting.

Potato leaflets were torn in half along the midrib, and half were provided to two 4th instars caged in small Petri plate arenas. The other half of each leaflet was immediately dried for alkaloid extraction. Beetles were allowed to feed and excrete for 24 hr, at which point the partly-eaten leaves and all the excrement in the Petri plate were collected and dried for extraction. If the beetles excrete all the alkaloids they ingest, and assuming they feed and excrete at fairly constant rates from one day to the next, the quantity of alkaloids in the uneaten leaflet half should be approximately equal to the quantity in the fed-on leaflet half plus excreta collected after feeding 24 hr. Leaflets from a total of 6 pairs of beetles were tested. Paired *t*-tests (Statgraphics Plus 5.0) were used to compare the amount of alkaloids in uneaten vs. fed-on leaflet halves plus excreta.

HPLC Analysis. α-Solanine and α-chaconine constitute at least 95% of the glycoalkaloids in commercial potato varieties (Friedman and McDonald, 1997) such as Russet Burbank, so they are the two alkaloids examined here. Beetles and potato foliage were assayed for solanine and chaconine according to methods based on Brown et al. (1999), and described in Armer et al. (in press). The same methods were also used to check for the presence of the aglycone solanidine (the sterol backbone to which three related sugars are added to create solanine and chaconine). Solanine, chaconine, and solanidine standards purchased from Sigma (St. Louis, MO) were used to calibrate the results.

RESULTS AND DISCUSSION

Neither solanine nor chaconine was found in any of the field-collected seven larval beetle or four adult beetle samples, or in the hemolymph sample. However, solanine and chaconine were both found in the excrement sample at levels approximately equal to that found in leaves of the same dried weight. The dried excrement contained 8.52 mg solanine and 11.08 mg chaconine per 100 g excrement. The leaf tissues upon which the CPB fed contained an average of 5.24 ± 3.99 mg (mean $\pm$ SD) of solanine and 19.61 ± 13.49 mg of chaconine per 100 g dried foliage (Armer et al., in press).

No difference in solanine quantities ($t_{10} = 2.14$, $P = 0.09$) was found between potato leaflet halves that had been analyzed without being fed on as compared to samples of fed-on leaflets and the wastes excreted during the 24-hour feeding period (Figure 1). Similarly, no differences were found in chaconine quantities (ln transformed data; $t_{10} = 1.22$, $P = 0.28$) in unfed-upon vs. fed-upon leaflet halves plus excreta (Figure 1).

The HPLC methods used provided a detection limit well below 1 ppm for both solanine and chaconine. Some chrysomelids successfully sequester alkaloids from host plants that have far lower host plant alkaloid concentrations than those found here in potato foliage. The CPB examined here did not show evidence

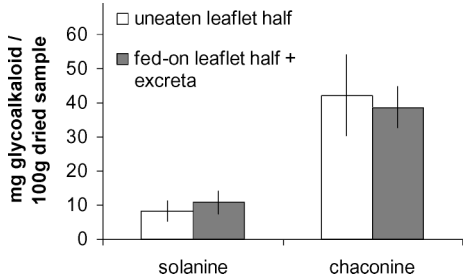

FIG. 1. Glycoalkaloids present in potato leaflet halves that were either intact or fed upon by Colorado potato beetle fourth instars. Two CPB larvae fed for 24 hours on leaflet halves in each of six arenas. The fed-upon samples included both the leaflet pieces remaining, as well as the excrement produced during the 24 hours. Bars indicate the SD. No differences in alkaloid levels were found between unfed leaflet halves and fed-on leaflet halves + excreta after feeding (paired *t*-test).

of containing any alkaloids. The beetles almost certainly had alkaloids in their guts, but the alkaloid level was below the detection limit, and they clearly do not sequester alkaloids in the same manner as some other chrysomelid species.

The aglycone solanidine cannot be extracted and/or quantified utilizing the HPLC methods applied to solanine and chaconine. In a total of 9 measurements of samples with solanidine standard in concentrations of 1–100 ppm, no peak was visible to indicate the presence of the compound. Additionally, a spike of 100 ppm solanidine was added to a CPB extract in a 50:50 mix, and no peak was visible in that spiked sample.

Clearly, solanine and chaconine are not found in adult or 4th instar CPB in appreciable amounts. The beetles might metabolize solanine and chaconine to less toxic forms by the sequential hydrolysis of the three sugars attached to the aglycone solanidine (Friedman and McDonald, 1997). However, the split-leaf assay suggests that approximately equal amounts of active alkaloids are found in the excreta as in the food, and that little or no glycoalkaloids are metabolized to the less toxic aglycone form in the CPB. Rothschild (1972) suggested that solanaceous foliar glycoalkaloids were not sequestered by CPB, but the experiments to support her assertion were never published. The work presented here, therefore, appears to be the first to provide evidence that the CPB excretes, rather than sequesters or metabolizes, the alkaloids from its host plants.

The data presented suggest the CPB does not sequester alkaloids for defense against its natural enemies. How, then, does the beetle avoid attack by a broad range of potential natural enemies? Daloze and colleagues (1986) documented a toxic dipeptide in a defensive fluid secreted by the CPB when disturbed. The hemolymph of the beetles also contains a distasteful substance (Deroe et al., 1977), which may be the same dipeptide as that found in the secretions.

Hsiao and Fraenkel (1969) identified a neurotoxic protein, called leptinotarsin, in the hemolymph of several *Leptinotarsa* species. Armer (2003) found that a hemolymph protein of the same molecular size as leptinotarsin was toxic to the symbiotic bacteria of the entomopathogenic nematode *Heterorhabditis marelatus* Liu & Berry. Thus, at least one pathogenic microorganism cannot develop properly within the CPB because of the presence of a defensive protein in the CPB. However, leptinotarsin was toxic only when injected into, but not when fed to, arthropods and mice (Hsiao and Fraenkel, 1969), and thus was probably not the toxin to which the aposematic coloration alerts predators.

Glycoalkaloids are present in the gut of the CPB as the insect feeds, and may be one toxin of which the beetle's aposematism warns. The few specialist natural enemies of the CPB appear to have developed behaviors to avoid contacting alkaloids in the gut of their host. The parasitic tachinid fly *Myiopharus doryphorae* (Riley) deposits neonate larvae into second- and third-instar CPBs, but the parasite larva does not begin to feed actively until the CPB larva has ceased feeding, entered the soil as a prepupa, and has voided its gut prior to pupation (Armer, unpublished data). Once the gut is emptied, the tachinid can feed throughout the host tissues without the risk of rupturing the gut and encountering toxic alkaloids. Heteropteran predators such as the pentatomid *Perillus bioculatus* (F.) may be able to avoid ingesting gut contents through feeding on select tissues. However, *P. bioculatus* has reduced longevity and fecundity when fed exclusively on CPB compared to other hosts (Armer, unpublished), and may be negatively affected by toxic compounds in the hemolymph.

Pasteels et al. (1990) discuss how sequestration can develop in an insect feeding on a toxic plant, and Rothschild (1972) points at possible evolutionary pathways of the relationship of aposematic coloration to toxin sequestration. In chrysomelid chemical defenses, autogenous production of toxins appears to be the basal state that allows the beetle to develop the pathways and enzymes necessary to sequester toxins from its host plant in the more derived condition. Aposematism develops independently of the ability to sequester host plant compounds, but enhances the benefits of sequestration. The CPB apparently fits into the primitive state, as its distinctive orange-and-black coloration appears to warn predators of autogenous toxins rather than glycoalkaloids sequestered from its host plants. Why the beetle does not make use of its host's alkaloids remains unanswered; it may be that phylogenetic constraints limit the beetle's ability to sequester the alkaloids. Or, it is possible that the dipeptides discussed by Daloze et al. (1986) provide all the defenses necessary, and developing the ability to sequester host alkaloids would be an energetically demanding evolutionary step not worth taking.

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ANALYSIS OF THE INSECT *os-d*-LIKE GENE FAMILY

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Abstract—Insect OS-D-like proteins, also known as chemosensory (CSP) or sensory appendage proteins (SAP), are broadly expressed in various insect tissues, where they are thought to bind short to medium chain length fatty acids and their derivatives. Although their specific function remains uncertain, OS-D-like members have been isolated from sensory organs (including the sensillum lymph in some cases), and a role in olfaction similar to that of the insect odorant binding proteins (OBP) has been suggested for some. We have identified 15 new OS-D-like sequences: four from cDNA clones described herein and 11 from sequence databases. The *os-d*-like genes from the *Anopheles gambiae*, *Apis mellifera*, *Drosophila melanogaster*, and *Drosophila pseudoobscura* genomes typically have single, small introns with a conserved splice site. Together with all family members entered on GenBank, a total of 70 OS-D-like proteins, representing the insect orders Diptera, Dictyoptera, Hymenoptera, Lepidoptera, Orthoptera, and Phasmatodea, were analyzed. A neighbor joining distance phenogram identified several protein similarity classes that were characterized by highly conserved sequence motifs, including (A) N-terminal YTTKYDN(V/I)(N/D)(L/V)DEIL, (B) central DGKELKXX(I/L)PDAL, and (C) C-terminal KYDP. In contrast, three similarity classes were characterized by their diversion from these conserved motifs. The functional importance of conserved amino acid residues is discussed in relation to the crystal and NMR structures of MbraCSPA6.

Key Words—OS-D-like, chemosensory protein (CSP), sensory appendage protein (SAP), insect, *Choristoneura fumiferana*, Lepidoptera.

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INTRODUCTION

The insect *os-d*-like gene family is represented by a group of small, highly soluble proteins with hydrophobic binding pockets. Named after the founding member that was cloned from the olfactory segment of *Drosophila melanogaster* antennae, they have also been referred to as chemosensory proteins (CSPs) and sensory appendage proteins (SAPs) based upon their association with sensory organs. The crystal and NMR structure of MbraCSPA6 from the moth *Mamestra brassicae* reveals a globular structure composed of six amphiphatic α-helices that surround a hydrophobic binding pocket, and two disulfide bonds that form α–α loops (Lartigue et al., 2002; Mosbah et al., 2003). OS-D-like proteins have been compared to insect odorant binding proteins (OBPs) that are similar in size, solubility, and overall structure (OBPs have six amphiphatic α-helices, joined by three disulphide bonds, that surround a hydrophobic binding pocket) (Rothemund et al., 1999; Sandler et al., 2000; Lee et al., 2002). In many (but not all) cases, OBPs are specifically expressed in the hydrophyllic sensillum lymph that surrounds olfactory neurons (for example, Galindo and Smith, 2001; Shanbhag et al., 2001; Vogt et al., 2002**)** where they are involved in the transport of hydrophobic odorants. In contrast, OS-D-like proteins are broadly expressed in various tissues (Kitabayashi et al., 1998; Picimbon et al., 2000, Jacquin-Joly et al., 2001), including sensillum lymph in some cases (Angeli et al., 1999; Nagnan-Le Meillour et al., 2000; Monteforti et al., 2002). Some OS-D-like proteins bind short to medium chain length fatty acid derivatives with low specificity (Nagnan-Le Meillour et al., 2000; Jacquin-Joly et al., 2001; Briand et al., 2002; Lartigue et al., 2002; Campanacci et al., 2003), and a more general physiological function relating to the transport/solubility of hydrophobic ligands in various tissues has been proposed.

Herein, we have cloned four new *os-d*-like sequences, three from the Eastern spruce budworm, *Choristoneura fumiferana*, and one from the cabbage looper, *Trichoplusia ni*. To provide a theoretical framework for future studies, the protein sequences were analyzed within the context of the insect OS-D-like family as a whole. To accomplish this, we have expanded the number of known *os-d*-like sequences by identifying nine new members from the *D. pseudoobscura* and *Apis mellifera* genome sequencing projects, and two from a *Bombyx mori* expressed sequence tag (EST) database. Combined together with GenBank sequences from the *D. melanogaster* and *Anopheles gambiae* genomes, and cDNA from the insect orders Dictyoptera, Hymenoptera, Lepidoptera, Orthoptera, and Phasmatodea, a total of 70 OS-D-like proteins are analyzed herein.

METHODS AND MATERIALS

Nomenclature. New protein sequences were reported as olfactory segment-D (OS-D) like, due to homology with the founding member isolated from the antennae of *D. melanogaster* (McKenna et al., 1994). Subsequently, OS-D-like

proteins have been referred to as chemosensory proteins (Angeli et al., 1999), sensory appendage proteins (SAP) (Robertson et al., 1999), and most recently, pherokines (PHK) (Sabatier et al., 2003). Herein, we have used the nomenclature associated with each published sequence where available; or, the GenBank accession number is used to refer to unnamed sequences. Genes that we have identified from ongoing genome sequencing projects, and that have not yet been annotated, are referred to as conceptual genes (CG).

Molecular Cloning of os-d-Like cDNAs. Second instar *C. fumiferana* larvae were supplied from a colony continuously maintained by the Insect Production Unit at the Great Lakes Forestry Centre, Sault Ste. Marie, Ontario, Canada. Larvae were reared on artificial diet at 24°C and a 16:8 L/D photoperiod, through to pupation and adult emergence.

A cDNA library of *C. fumiferana* was constructed using the Uni-ZAP XR vector (Stratagene, La Jolla, CA) with mRNA isolated from larvae that were molting from the fifth to the sixth instar. cDNA clones were randomly selected and sequenced from the 5′ ends to generate ESTs. The ESTs were annotated based on Blastn and Blastx searches against the nonredundant GenBank database. Clones identified as *os-d*-like were further sequenced using forward and reverse sequencing primers (BRL), the Taq Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems), and an automatic DNA sequencer (model 310, Applied Biosystems Inc).

Total RNA was extracted in guanidium isothiocyanate from the head (including antennae, proboscis, and labial palps) and front tarsi of adult *C. fumiferana* (mixed male and female), and purified by acid phenol/chloroform extraction (Sambrook et al., 1989). First strand cDNA was synthesized from 5 μg of total RNA by using an anchored oligo dT primer: 5′ GCG CCG CGG CCG CT_{11} (CT)(ACG) 3′. Reaction conditions were 200 U SuperScript II reverse transcriptase in 1× 1st strand buffer (Gibco), 0.5 mM dNTP mix, 10 U cloned RNase inhibitor (Invitrogen), and 500 ng oligo dT primer in 20 μl total volume and a 50 min incubation at 42°C. For RT-PCR, a truncated dT primer (5′ GCG CCG CGG CCG CTT 3′) was used in combination with a redundant primer designed to match a conserved amino acid sequence. The *os-d*-like redundant primer A (5′ TGC A[CG]T CCT GA[CG] GG[ACGT] AAA GA[AG] CT[CT] AA 3′) was designed to match the conserved amino acid sequence C(T/S/A)(P/A/D/V)(D/E)(G/A)KELK. An alignment of cDNA sequences was used to estimate codon preference and to reduce the redundancy of the primer. PCR reaction conditions were 2 μl of 1st cDNA, 1.25 U Taq DNA polymerase in 1× Taq buffer (Invitrogen), 2.0 mM $MgCl_2$, 0.4 mM truncated oligo dT primer, 4 mM redundant primer, in 50 μl total volume. PCR reactions were amplified using a Geneamp 2400 thermo-cycler (Perkin-Elmer) as follows: one cycle of 2 min at 94°C, 30 cycles of 30 sec at 94°C, 40 sec at 50°C, 1 min at 72°C, and finally one cycle of 7 min at 72°C. PCR products were gel purified using Qiex beads (Qiagen) and blunt-end cloned into pBluescript II KS (GenBank # ARBL2KSM) cut with the EcoRV restriction enzyme (Invitrogen).

Plasmid clones were sequenced in both directions. A PCR product was also cloned from *T. ni* as outlined above (insects were supplied from a colony continuously maintained on artificial diet at the laboratory of Dr M. Isman).

Identification of OS-D-like Proteins from Sequence Databases. GenBank nonredundant and EST databases were searched using PSI Blast, Blastp with a PHI pattern for conserved Cys residues ($CX_6CX_{16-19}CX_2C$), and Blastn (Altschul et al., 1997). All known OS-D-like sequences were used as query sequences. EST databases available on the World Wide Web (WWW) were also searched: SilkBase (www.ab.a.u-tokyo.ac.jp/silkbase/), and the Honey Bee Brain EST Project (www.titan.biotec.uiuc.edu/bee/honeybee_project.htm). *D. pseudoobscura* and *A. mellifera* genome trace files were downloaded from the NCBI trace archive and searched for nucleotide sequences that coded for OS-D-like protein sequences using a stand alone implementation of tBlastn. Individual trace files were assembled (minimum $5\times$ coverage where possible) into contiguous sequences containing complete open reading frames (ORF).

Gene Predictions and Conceptual Translation. Contiguous sequences were used as tBlastn queries against the GenBank nonredundant database to identify general exon/intron boundaries by homology with known *os-d*-like ORFs. Intron splice sites were identified by the conserved intron start (GT) and end (AG) sequences. Coding regions were combined to form conceptual ORFs that were translated using the standard genetic code. In the case of cDNA sequences, and genes without introns, the ORF was identified by translating a continuous sequence between a start and stop codon. Conceptual protein sequences were then assessed for the characteristic features of the OS-D-like protein family: four invariant Cys residues, sequence homology with known members, and a length of 110–160 amino acid residues.

Protein Similarity Groups. Putative signal peptides were identified by comparison with mature OS-D-like proteins reported in the literature. For analysis (and calculation of protein sequence identities), proteins were truncated at a point 28 residues prior to the first conserved Cys residue to eliminate the highly variable signal peptides. Truncated protein sequences were aligned using CLUSTL_X (Thompson et al., 1997); an unrooted neighbor joining distance phenogram was constructed with bootstrap support using PAUP (Phylogenetic Analysis Using Parsimony) 4.0 Beta Version 10 Windows interface (Swofford, 2002).

RESULTS

Cloning os-d-Like cDNAs. Four new *os-d*-like cDNAs were cloned. Two unique sequences, *CfumAY426538* & *CfumAY426539* (Figure 1), were identified from a *C. fumiferana* larval cDNA library by a tBlastn search of random sequences; both deduced amino acid sequences have four invariant Cys residues ($CX_6CX_{18}CX_2C$) consistent with the OS-D-like protein family (Figure 2).

CfumAY426538

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TTATCCTGTCAACTCTGGTGGTCCTGGGCCGTTGGGCAAGGGACGTATACGGCTGAGAAT
L  S  C  Q  L  W  W  S  W  A  V  G  Q  G  T  Y  T  A  E  N
GACGACTTGGACATCGATGGCATCGTGAAAGATCCGAAGAAGCTGCAAGAATGGTTCGGC
D  D  L  D  I  D  G  I  V  K  D  P  K  K  L  Q  E  W  F  G
TGCTTCGTTGACAAGAGCCCTTGCGATAACGTGCAGCTTAGCTTCAAAGCTGACATGCCA
C  F  V  D  K  S  P  C  D  N  V  Q  L  S  F  K  A  D  M  P
GAAGCAATCCGGGAAGCTTGCGCGAAATGCACCACGGCACAGAAGGGAATCTTGAAGAAA
E  A  I  R  E  A  C  A  K  C  T  T  A  Q  K  G  I  L  K  K
TTCCTCGTAGGCCTCGAGGAGAAGGCCCCAGCTGATTACGAAGTGTTCAAGAAGAAATAC
F  L  V  G  L  E  E  K  A  P  A  D  Y  E  V  F  K  K  K  Y
GACTCTGAAAACAAATACATTGAGCCGCTTAAAAAAGCTATTGCATAAAATCGGGGTTAA
D  S  E  N  K  Y  I  E  P  L  K  K  A  I  A  *
TACATGTACTAATAATACTCTCCCTGCCAACAATATAAGGCTTTCCAAGTTCAAGTAGCC
ATGAGAGTTATGAGAGCGCCGTGGATCACAAAAATAAATAAAAATGGGCACAGCCTGAAT
ATAAAGAACTTCGAAGATGCTTGTGTCTGT
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CfumAY426539

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GACTCGCCCGAAACCCGTACCAGGCAATCATAACCATGAAGAGTATACTCTATCTCGTGC
                                  M  K  S  I  L  Y  L  V  L
TGACGGTGGTGGTGACCTGCTCGGCGCAGCAACAGTACTACAACAACCGATATGACAACC
  T  V  V  V  T  C  S  A  Q  Q  Q  Y  Y  N  N  R  Y  D  N  L
TCAACGCTGACTCTATCGTGCAGAACGAACGAGTTCTGCTCGCCTACTACAAGTGCGTTA
  N  A  D  S  I  V  Q  N  E  R  V  L  L  A  Y  Y  K  C  V  M
TGGACAAAGGACCTTGCACTAAGGACGGAAAAAACTTCAAACGCGTACTACCAGAGACGC
  D  K  G  P  C  T  K  D  G  K  N  F  K  R  V  L  P  E  T  L
TGTCAACAGCCTGCGCCCGCTGCTCCCCGAAGCAGAAAGGACTCGTACGCACCCTCCTTC
  S  T  A  C  A  R  C  S  P  K  Q  K  G  L  V  R  T  L  L  L
TCGGCATCAGGGTCAAAAGCGAGCCGCGTTTCAACGAACTTCTGGACAAATACGACCCCG
  G  I  R  V  K  S  E  P  R  F  N  E  L  L  D  K  Y  D  P  D
ACCGCTCCAACCGGGATGACCTGTACAAGTTCTTAGTCACCGGCAACTAAATTTAAATAC
  R  S  N  R  D  D  L  Y  K  F  L  V  T  G  N  *
CACCGCGAACCGGAATAGTTGAGCCACTCGCGCTATTCATAATCCAGCCGGGTTATATAC
TCGTAGTAAAATATCGTCATTAATAATACCCGAAAAGCATACGGCCAAATTACGAACATT
GGCATGTGGCCCAGCAATCCCGGTGCGCGACTGTA
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CfumAY426540

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GATACATTGCCTGATGCTTTGGAAAATGAGTGCAACAAATGCACGGAAAAACAGAAGTCG
D  T  L  P  D  A  L  E  N  E  C  N  K  C  T  E  K  Q  K  S
GGATCAGATAAGGTTATCAGGCATCTCGTTAACAAACGTCCCGAAATGTGGAAGGAGCTG
G  S  D  K  V  I  R  H  L  V  N  K  R  P  E  M  W  K  E  L
TCGGTGAAGTACGACCCTGATCATATCTATGAAGGCAGGTACAAGGACCAGATTGAGAAG
S  V  K  Y  D  P  D  H  I  Y  E  G  R  Y  K  D  Q  I  E  K
ATCAAGGCGTAGGAAGGGGAACTGATGTCTCCAAGGAGCACTGAAACTGTTGGACGTTTT
I  K  A  *
GAGATTGGTTTTCCTTGTTTGTAATAAACGTCCTTTTACCAT
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TniAY456191

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GAAACTCTTCCCGATGCTCTCGAACACGAGTGCGTCAAGTGTACGGAGAAACAGAAGTCT
E  T  L  P  D  A  L  E  H  E  C  V  K  C  T  E  K  Q  K  S
GGCTCAGAGAAGGTGATCAGACACTTGGTGAACAGGCGTCCGGACTTGTGGAAGGAGCTG
G  S  E  K  V  I  R  H  L  V  N  R  R  P  D  L  W  K  E  L
GCGACCAAGTATGACCCTGACAACATCTACCAAGACAGATACAAGGACAAGATCCAAGCT
A  T  K  Y  D  P  D  N  I  Y  Q  D  R  Y  K  D  K  I  Q  A
GCCAAGGGCCAGTAACGTAGCAAGACTTAGCTGTGAACGTTCCACAGCTAGTGAATAGGT
A  K  G  Q  *
GATGGAAGTGTAAATTGTGATTAAAAACGCAGTTACCAAAGCTTTAATTGGAGTTTATTT
CAATAAATTAATTTGTATCC
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FIG. 1. Nucleic acid sequence and conceptual translation of cDNA isolated from a *C. fumiferana* larval cDNA library (*CfumAY426538* and *CfumAY426539*) and cloned using RT-PCR products amplified from *C. fumiferana* and *T. ni* using a redundant primer based on the conserved amino acid sequence C(T/S/A)(P/A/D/V)(D/E)(G/A)KELK (*CfumAY426540* and *TniAY456191*). Start and stop codons are underlined; conserved cysteine residues are bolded and underlined.

CfumAY426539 contains the complete ORF, while the amino acid sequence of *CfumAY426538* begins within the putative signal peptide. Both sequences are unique, sharing more homology with *A. gambiae* OS-D-like proteins than with other lepidopteran members (Blastp scores, e-10 and e-14, respectively).

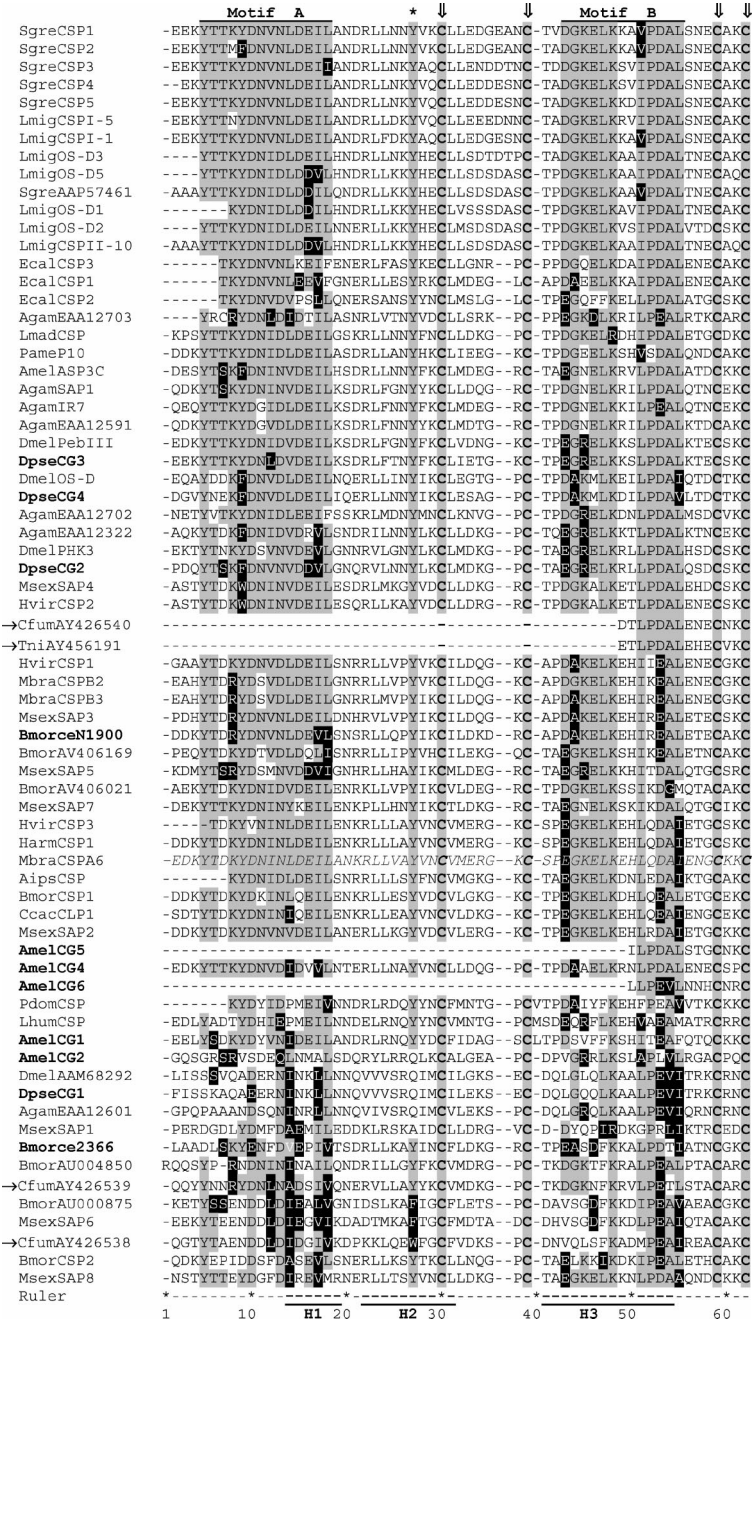
A redundant primer corresponding to the conserved amino acid sequence C(T/S/A)(P/A/D/V)(D/E)(G/A)KELK was used to amplify cDNA from a specific lepidopteran subclass. Two cDNA fragments, approximately 300 bp in length, were cloned from *C. fumiferana* and *T. ni*, and found to encode peptides of 63 and 64 amino acids that are most similar by Blastp search to MsexSAP4 and HvirCSP2 (Figures 1 and 2, Table 1). The clones, termed *CfumAY426540* and *TniAY456191*, encode the C-terminal halves of the protein.

Identification of OS-D-Like Proteins from Sequence Databases. In total, 66 OS-D-like sequences representing the insect orders Diptera, Dictyoptera, Hymenoptera, Lepidoptera, Orthoptera, and Phasmatodea were identified from sequence databases: 55 from GenBank (identified by a PSI-blast search using all known OS-D-like sequences, and a Blastp using the conserved Cys spacing motif $CX_{6–8}CX_{16–19}CX_2C$ as a PHI pattern), nine from the ongoing *D. pseudoobscura* and *A. mellifera* genome sequencing projects (constructed herein from trace files), and two from a *B. mori* EST database (Silkbase, www.ab.a.u-tokyo.ac.jp/silkbase/) (Table 1).

Several *A. gambiae* OS-D-like proteins identified from GenBank (Table 1) were not entirely correct in their sequence. AgamEAA12601, AgamEAA12322, AgamEAA12353, AgamEAA12338, and AgamEAA12591 all have additional peptide sequence at the N-terminus, and AgamEAA12702 is missing the C-terminal half. Genomic contigs were downloaded from the Ensembl database (www.ensembl.org/Anopheles_gambiae/), and the genes and their translation products predicted manually as outlined in the Methods and Materials section. EST sequences that code for AgamEAA12353 and AgamEAA12338 have been reported: AgamSAP1 (AAL84186; Biessmann et al., 2002) and AgamIR7 (AF283263; Oduol et al., 2000), respectively.

The sequence of *Agameaa12703* is anomalous. The coding region of the first exon (bordered at the 5′ end by a stop codon) is incomplete and does not begin

FIG. 2. Alignment of the OS-D-like protein family. Shaded regions indicate conserved motifs, with conservative substitutions highlighted as white text on a black background. Aromatic residues at positions 27, 85, and 98 are marked with an *; the four conserved Cys residues are in bold text and are marked by an ⇓. The sequence of MbraCSPA6, for which the crystal and NMR structures have been solved (Lartigue et al., 2003; Mosbah et al., 2003), is italicized. Underlined sections of the ruler correspond to the six helices of MbraCSPA6. An → signifies sequences translated from cDNA clones described herein; the names of new sequences identified herein from sequence databases are bolded.


Motif A
Motif B
SgreCSP1
SgreCSP2
SgreCSP3
SgreCSP4
SgreCSP5
LmigCSPI-5
LmigCSPI-1
LmigOS-D3
LmigOS-D5
SgreAAP57461
LmigOS-D1
LmigOS-D2
LmigCSPII-10
EcalCSP3
EcalCSP1
EcalCSP2
AgamEAA12703
LmadCSP
PameP10
AmelASP3C
AgamSAP1
AgamIR7
AgamEAA12591
DmelPebIII
DpseCG3
DmelOS-D
DpseCG4
AgamEAA12702
AgamEAA12322
DmelPHK3
DpseCG2
MsexSAP4
HvirCSP2
→CfumAY426540
→TniAY456191
HvirCSP1
MbraCSPB2
MbraCSPB3
MsexSAP3
BmorceN1900
BmorAV406169
MsexSAP5
BmorAV406021
MsexSAP7
HvirCSP3
HarmCSP1
MbraCSPA6
AipsCSP
BmorCSP1
CcacCLP1
MsexSAP2
AmelCG5
AmelCG4
AmelCG6
PdomCSP
LhumCSP
AmelCG1
AmelCG2
DmelAAM68292
DpseCG1
AgamEAA12601
MsexSAP1
Bmorce2366
BmorAU004850
→CfumAY426539
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MsexSAP6
→CfumAY426538
BmorCSP2
MsexSAP8
Ruler
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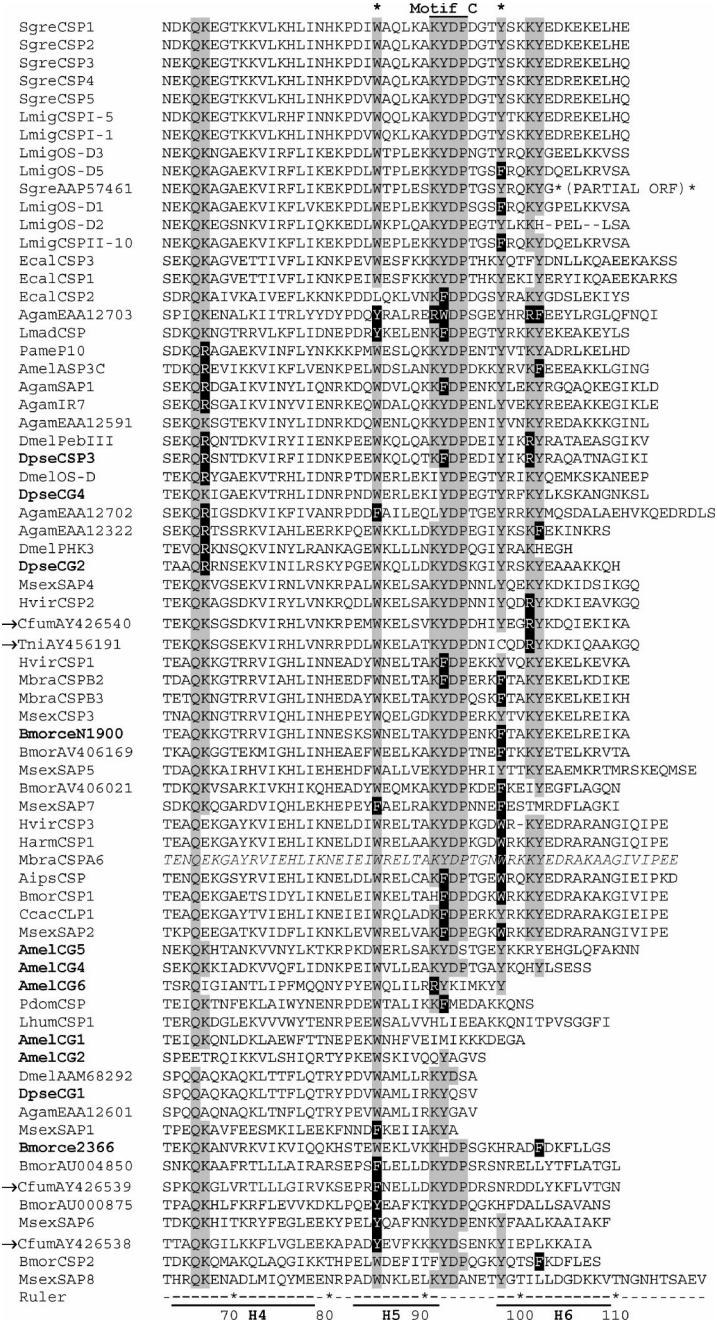

FIG. 2. Continued.

TABLE 1. LIST OF OS-D-LIKE PROTEINS

Order: Family	Species	Protein name[a,b]	Accession number	Expression[c]	Binding studies
O. Diptera		DmelPEBIII	AAF47140	L La P W (Sabatier et al., 2003)	
F. Drosophilidae	*Drosophila melanogaster*	DmelOS-D*	AAA21358	A (McKenna et al., 1994)	
		DmelAAM68292	AAM68292		
		DmelPHK3	AAF47307	E L P (Sabatier et al., 2003)	
	D. pseudoobscura	**DpseCG1 to 4**			
F. Culicidae		AgamEAA12601	EAA12601		
	Anopheles gambiae	AgamEAA12322	EAA12322		
		AgamSAP1	EAA12353	A B (Biessmann et al., 2002)	
		AgamIR7	EAA12338	B (Oduol et al., 2000)	
		AgamEAA12591	EAA12591		
		AgamEAA12703	EAA12703		
		AgamEAA12702	EAA12702		
O. Hymenoptera	*Apis mellifera*	**AmelCG1** & **2** and **4** to**6**			
F. Apidae		AmelASP3C	AAN59784	A (Briand et al., 2002)	Briand et al., 2002
F. Formicidae	*Linepithema humile*	LhumCSP	AAN01363	A (Ishida et al., 2002)	
F. Vespidae	*Polistes dominulus*	PdomCSP1	AAP55719		
O. Lepidoptera		BmorCSP1	AAM34276	A A_b H L T_h (Picimbon et al., 2000)	
F. Bombycidae	*Bombyx mori*	BmorCSP2	AAM34275	A A_b H L T_h (Picimbon et al., 2000)	
		BmorAV406169	AV406169		
		Bmorce2366	ce2366		
		BmorceN1900	ceN1900		
		BmorAU004850	AU004850		
		BmorAV406021	AV406021		
		BmorAU000875	AU000875		
F. Noctuidae	*Agrotis ipsilon*	AipsCSP	AAP57460		
	Helicoverpa armigera	HarmCSP	AAK53762		
	Heliothis virescens	HvirCSP1	AAM77040	A L T_h (Picimbon et al., 2001)	

TABLE 1. CONTINUED

Order: Family	Species	Protein name[a,b]	Accession number	Expression[c]	Binding studies
		HvirCSP2	AAM77041	A H L T_h(Picimbon et al., 2001)	
		HvirCSP3	AAM77042	A L (Picimbon et al., 2001)	
	Mamestra brassicae	MbraCSPA6**	AAF71289	A P_r P_g (Nagnan-Le Meillour et al., 2000)[d]	
		MbraCSPB2	AAF19653	P_r (Nagnan-Le Meillour et al., 2000)	
		MbraCSPB3	AAF71290	A P_g (Jacquin-Joly et al., 2001)	
	Trichoplusia ni	→TniAY456191	AY456191		
F. Pyralidae	*Cactoblastis cactorum*	CcacCLP1	AAC47827	L_a (Maleszka and Stange, 1997)	
F. Sphingidae	*Manduca Sexta*	MsexSAP1	AF117574	A (Robertson et al., 1999)	
		MsexSAP2	AF117592	A (Robertson et al., 1999)	
		MsexSAP3	AF117585	A (Robertson et al., 1999)	
		MsexSAP4	AF117599	A (Robertson et al., 1999)	
		MsexSAP5	AF117594	A (Robertson et al., 1999)	
		MsexSAP6	BE015509		
		MsexSAP7	CA798851		
		MsexSAP8	CA798912		
F. Tortricidae	*Choristoneura fumiferana*	→CfumAY426538	AY426538		
		→CfumAY426539	AY426539		
		→CfumAY426540	AY426540		
O. Dictyoptera					
F. Blaberidae	*Leucophaea maderae*	LmadCSP	AAM77025		
F. Blattidae	*Periplaneta americana*	PameP10	AAB84283	A H (Kitabayashi et al., 1998)	
O. Orthoptera	*Locusta migratoria*	LmigOS-D1	CAB65177	A L (Picimbon et al., 2000)	
F. Acrididae		LmigOS-D2	CAB65178	A L (Picimbon et al., 2000)	
		LmigOS-D3	CAB65179	A L (Picimbon et al., 2000)	
		LmigOS-D5	CAB65181	A L (Picimbon et al., 2000)	

		LmigCSPI-1	AAO16783	A W (Ban et al., 2003)	
		LmigCSPI-5	AAO16787	A W (Ban et al., 2003)	
		LmigCSPII-10	AAO16793	A W (Ban et al., 2003)	Ban et al., 2003
	Schistocerca gregaria	SgreCSP1**	AAC25399	A T L_a(Angeli et al., 1999)	
		SgreCSP2**	AAC25400	A T L_a(Angeli et al., 1999)	
		SgreCSP3**	AAC25401	A T L_a(Angeli et al., 1999)	
		SgreCSP4**	AAC25402	A T L_a(Angeli et al., 1999)	Ban et al., 2002
		SgreCSP5**	AAC25403	A T L_a(Angeli et al., 1999)	
		SgreAAP57461	AAP57461		
O. Phasmatodea		EcalCSP1	AAD30550	A C T (Marchese et al., 2000)	
F. Phasmatidae	*Eurycantha calcarata*	EcalCSP2	AAD30551	A T (Marchese et al., 2000)	
		EcalCSP3	AAD30551	A T (Marchese et al., 2000)	

[a] → signifies sequences translated from cDNA clones described herein, the names of new sequences identified herein from sequence databases are bolded. [b] * indicates expression associated with sensilla, ** protein has been found in sensillum lymph. [c] A = antennae, A_b = abdomen, B = body, C = cuticle, E = embryo, H = head, L = legs, L_a = labrum or labial palp, P = pupae, P_g = pheromone gland, P_r = proboscis, T = tarsi, T_h = thorax, W = wings. [d] MbraCSPA6; the 3-D structure has been solved, and several binding studies conducted (Bohbot et al., 1998; Campanacci et al., 2003; Lartigue et al., 2002; Mosbah et al., 2003; Nagnan-Le Meilour et al., 2000).

with an AUG/GUG start codon. The coding region of the second exon (terminated by a stop codon) is unusually long. Translation of *Agameaa12703* results in a protein with 335 residues, the first 112 of which are homologous to OS-D-like proteins (beginning within the putative signal peptide) (Figure 2). The additional protein sequence is not homologous to any entered on GenBank, and it contains an unusually long stretch of threonine (Thr) residues. No sequence errors could be detected. MsexSAP5 (Table 1), translated from cDNA, is similarly anomalous. It has an extended C-terminus consisting of six imperfect repeats of a 14 amino acid motif, making the protein length 231 residues in total (Robertson et al., 1999).

Four *os-d*-like genes were identified from the unassembled *D. pseudoobscura* genome; the translated proteins are referred to as DpseCG1-4 (Figure 2). Each gene was constructed using the following NCBI trace archive files: *Dpsecg1* (149079898, 154972476, 155144441, 155211255, and 155212923); *Dpsecg2* (151302570, 153344964, 155268885, 156510153, and 158760462); *Dpsecg3* (153386764, 155134102, 167465427, 168214922, and 168250005); and *Dpsecg4* (149219044, 159241862, 168274023, 169327986, and 182677706).

Six genes were identified by tBlastn searches of the unassembled *A. mellifera* genome; the translated proteins are referred to as AmelCG1-6 (Figure 2). Each gene was constructed using the following trace files: *Amelcg1* (165820474, 166380969, and 166555471); *Amelcg2* (165857634, 165954160, 171052956, 173768694, and 173904508); *Amelcg3* (159575920, 165888936, 166568511, 173284880, and 174014423); *Amelcg4* (161249969, 166197062, 173383798, 173396536, and 173485622); *Amelcg5* (160832583, 161246793, 165954381, and 174042462); and *Amelcg6* (166299781, 166383082, and 174048292). *Amelcg1* and 4 contain complete ORFs. Because of incomplete sequence coverage, *Amelcg3* begins within the N-terminal coding region, and *Amelcg5* and 6 begin within the C-terminal coding region. An OS-D-like protein identified from the antennae of *A. mellifera* (AmelASP3C, Briand et al., 2002, Table 1) is almost identical to the partial sequence of AmelCG3 (83/85 identities); for further analysis, AmelASP3C was used in place of AmelCG3. A start codon could not be identified for *Amelcg2*, however, sequence coverage in this region was poor.

Genomic Organization and Intron Structure. All *A. gambiae os-d*-like genes are located within a 120 Kb section of chromosome 3R, and four are clustered within a 20 Kb region (Figure 3, Ensembl database, www.ensembl.org/Anopheles_gambiae/). *DmelAAM68292* and *DmelPhk3* are located within 5 Kb of each other, and approximately 900 Kb from *DmelPebIII* on chromosome 2R. *Dmelos-d* is located on chromosome 3L (Figure 3, Flybase, www.flybase.bio.indiana.edu/). An alignment of the *D. melanogaster* and *D. pseudoobscura* genomes (www.pipeline.lbl.gov/) places *Dpsecg1-4* in the same genomic locations as *DmelAAM68292*, *DmelPhk3*, *DmelPebIII*, and *Dmelos-d*, respectively.

Three *A. gambiae* and four *Drosophila* genes lack introns. All other known *os-d*-like genes from *A. gambiae*, *A. mellifera*, *D. melanogaster*, and *D.*

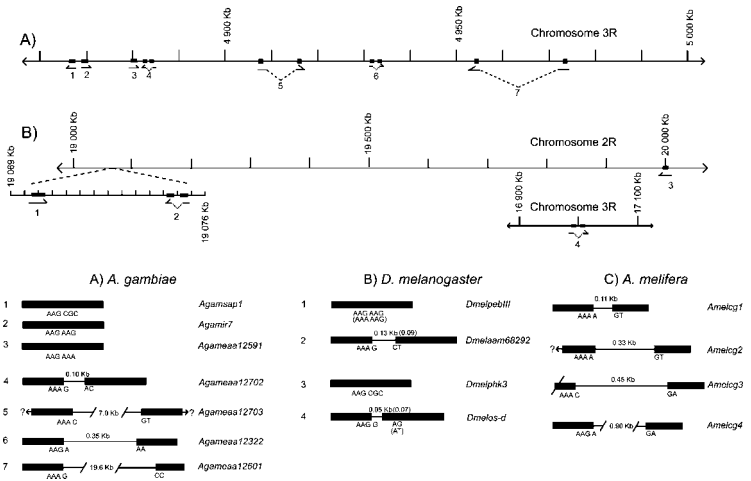

FIG. 3. Genomic organization and intron structure of *os-d*-like genes. (A) *A. gambiae* (Ensembl database, www.ensembl.org/Anopheles_gambiae/); (B) *D. melanogaster* (Flybase, www.flybase.bio.indiana.edu/); and (C) *A. mellifera*. A solid block (▬) denotes coding regions with homology to OS-D-like proteins; an →? indicates that a start and/or stop codon was not identified; and ▬⚑ indicates incomplete sequence resulting in translation of a partial coding region. Introns are represented by a line joining two adjacent coding regions. Codons flanking the conserved intron splice site (located one nucleotide past a conserved Lys codon, AAA/G), and the equivalent position in genes lacking introns, are listed below the coding region/intron boundary. The location and intron structure of *D. pseudoobscura* genes is virtually identical to that of *D. melanogaster* based on an alignment of the two genomes (www.pipeline.lbl.gov/); therefore, only the differences have been noted (bracketed values following intron sizes and/or intron boundary sequence).

pseudoobscura have single, typically small introns (Figure 3). The introns of *A.gamEAA12703* and *A.gamEAA12702* are significantly larger, approximately 7.0 and 19.6 Kb, respectively. The intron splice site is conserved, always located one nucleotide past a conserved lysine (Lys) codon (Figure 3) that corresponds to amino acid position 48 in Figure 2. This Lys residue is conserved in all members of the OS-D-like protein family identified to date, with only two exceptions: LmadCSP and MsexSAP1 have a conservative substitution of an arginine (Arg) for the Lys (Figure 2). The conserved splice site, therefore, may be a general characteristic of the *os-d*-like gene family. The genes without introns have likely lost them secondarily, since all retain the conserved sequence associated with the splice site (Figures 2 and 3).

Protein Similarity Groups. The protein alignment in Figure 2 indicates that many members of the OS-D-like protein family, representing at least six insect orders, have conserved several sequence motifs, including (A) N-terminal YTTKYDN(V/I)(N/D)(L/V)DEIL, (B) central DGKELKXX(I/L)PDAL, and (C) C-terminal KYDP. In addition, aromatic residues at positions 27, 85, and 98 that may be functionally important (see discussion) are also highly conserved, along with residues at positions 66/67 (glutamine/lysine) and 101/102 (lysine/tyrosine). In contrast, the sequences of 17 OS-D-like proteins from the orders Diptera, Hymenoptera, and Lepidoptera (but not the Dictyoptera, Orthoptera, and Phasmatodea) clearly diverge from the conserved motifs A-C identified in Figure 2. In addition, they vary in the retention of, or diversion from, the two aromatic residues at positions 27 and 98 in three general categories: (A) both are retained, (B) position 98 has diverged, and (C) both positions have diverged, and these proteins are truncated at the C-terminus.

Ten protein similarity classes were identified using the neighbor joining method to construct an unrooted distance phenogram representing all known OS-D-like sequences (Figure 4). With the exception of two similarity classes from the Orthoptera, all are represented at, or higher than, the family level of taxonomy. Seven are characterized by retention of the conserved motifs A-C (Diptera Class 1 and 2, Lepidoptera Class 1–3, and Orthoptera Class 1 and 2), and three are characterized by diversion from these conserved motifs (Diptera/Hymenoptera Class 1, Hymenoptera Class 1, and Lepidoptera Class 4) (Figure 4). Of significance, each similarity class identified is homogenous with respect to retention of, or diversion from, the conserved motifs. The Hymneoptera/Diptera similarity class (Figure 4), a group that diverges from the conserved motifs, is represented by two different insect orders.

The grouping of Diptera Class 1 (Figure 4) is further supported by the fact that all the members (*Agamsap1*, *Agamir7*, *Agameaa12591*, *DmelpebIII*, and *Dpsecg3*) lack introns (Figure 3). The fact that *Agamsap1*, *Agamir7*, *Agameaa12591* are clustered closely together, share 75–79% amino acid identity, and group together with only one homologous *Drosophila* member, may indicate that they resulted

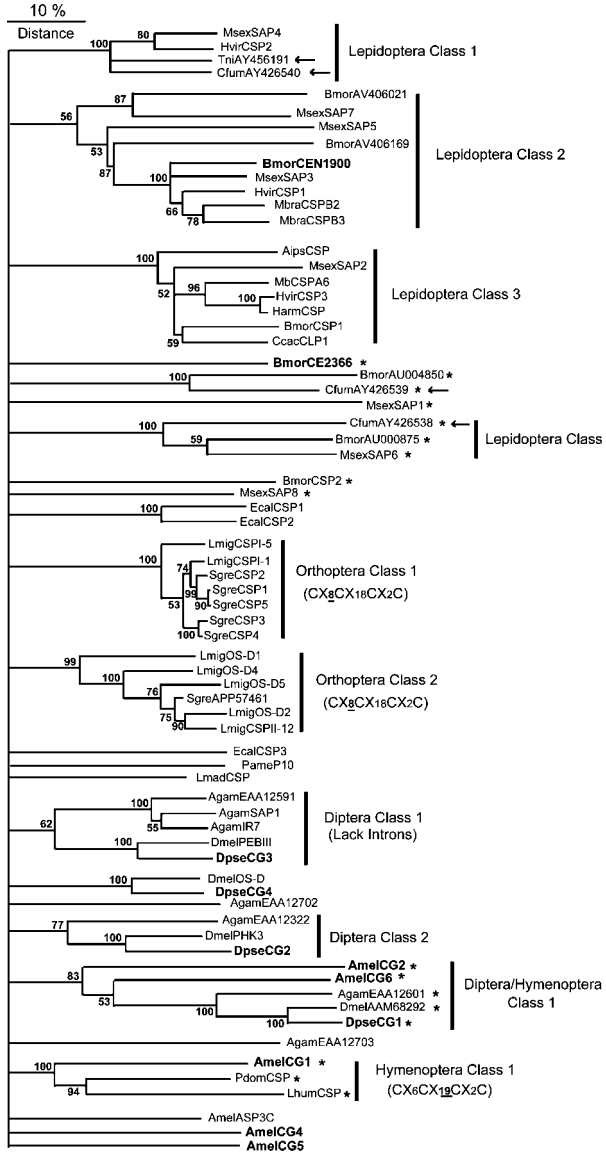

FIG. 4. Neighbor joining distance phenogram of all known OS-D-like protein sequences, collapsed to nodes with 50% or greater bootstrap support, $N = 1000$ replicates. Branch lengths are proportional and the scale represents percent sequence distance. An → indicates sequences translated from cDNA reported herein; new sequences identified herein from sequence databases are bolded; and an * is used to label sequences that diverge from conserved motifs A–C identified in Figure 2.

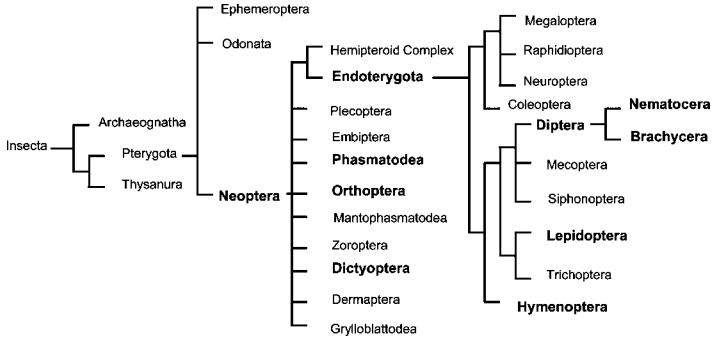

FIG. 5. Evolutionary tree of the Insecta (from The Tree of Life Web Project, www.tolweb.org/tree/phylogeny.html). Phyla from which OS-D-like proteins have been identified are represented in bolded text.

from gene duplication that occurred within the suborder Nematocera (*A. gambiae*), but not the suborder Brachycera (*Drosophila*), a taxonomic division that occurred early within the ancestral Diptera (Figure 5). However, it should be noted that members of Diptera Class 2 are mixed with regard to intron retention (*Dmelphk3*/*Dpsecg2* lack introns, while *Agameaa12322* has an intron), and further data are required to establish evolutionary relationships.

Diversion from the conserved Cys spacing pattern $CX_6CX_{18}CX_2C$ is uncommon, and when it does occur, it is associated with specific similarity classes. All Orthopteran OS-D-like proteins identified to date (representing a single taxonomic family) are characterized by the insertion of two additional residues between the first and second conserved Cys residues ($CX_{\underline{8}}CX_{18}CX_2C$) (Orthoptera Class 1 and 2, Figures 2 and 4). Hymenoptera Class 1, represented by three different taxonomic families, has an additional residue located between the second and third conserved Cys residues ($CX_6CX_{\underline{19}}CX_2C$). MsexSAP1 represents a single example of a deletion, between the second and third conserved Cys residues ($CX_6CX_{\underline{17}}CX_2C$) (Figure 2).

DISCUSSION

OS-D-like proteins share some features in common with insect OBPs (both are small, highly soluble proteins with hydrophobic binding pockets), but they do not share sequence homology, and they represent two distinct classes. An association with sensory organs, including the sensillum lymph in some cases, led to the hypothesis that OS-D-like proteins may be a new and different type of OBP (Bohbot et al., 1998; Angeli et al., 1999; Marchese et al., 2000; Nagnan-Le Meillour et al., 2000; Monteforti et al., 2002). *Dmelos-d*, the first to be discovered,

was cloned from the olfactory segment of adult *D. melanogaster* antennae using subtractive cDNA methods (McKenna et al., 1994). Subsequently, OS-D-like proteins were identified from the chemosensory organs (such as the antennae, labial palps, and proboscis) of several insect species (Table 1), including *Cactoblastis cactorum* (Maleszka and Stange, 1997), *Periplaneta americana*, *P. fuliginosa*, *Blattella germanica* (Picimbon and Leal, 1999), *Schistocerca gregaria* (Angeli et al., 1999), *Locusta migratoria* (Picimbon et al., 2000), *M. brassicae* (Nagnan-Le Meillour et al., 2000), *Manduca sexta* (Robertson et al., 1999), *Eurycantha calcarata* (Marchese et al., 2000), *A. mellifera* (Briand et al., 2002), *A. gambiae* (Biessmann et al., 2002), and *Polistes dominulus* (Ishida et al., 2002). The immuno-histological localization of OS-D-like proteins to the sensillum lymph that surrounds sensory neurons (Angeli et al., 1999; Nagnan-Le Meillour et al., 2000; Monteforti et al., 2002) supported an olfactory function for these proteins, along with the demonstration that an OS-D-like protein from antennae was able to bind pheromone components (Bohbot et al., 1998; Jacquin-Joly et al., 2001).

However, in contrast to a purely olfactory function, OS-D-like proteins have also been isolated from nonchemosensory organs (Table 1) (Kitabayashi et al., 1998; Picimbon et al., 2000, 2001; Jacquin-Joly et al., 2001), and in conjunction with evidence indicating a broad ligand binding specificity (Nagnan-Le Meillour et al., 2000; Jacquin-Joly et al., 2001; Briand et al., 2002; Lartigue et al., 2002; Campanacci et al., 2003), a more general physiological function relating to the transport of hydrophobic molecules in various tissues has been proposed. PameP10 was isolated from regenerating cockroach legs at a concentration 30 times greater as compared to mature legs, and was associated with the developing epidermis (Kitabayashi et al., 1998). *os-d*-Like genes are expressed generally in the thoraces, abdomen, legs, and heads of two Lepidopteran species (Picimbon et al., 2000, 2001), and MbraCSPA6 is expressed in the pheromone gland (Jacquin-Joly et al., 2001). Ligand binding studies indicate that AmelASP3C and MbraCSPA6 bind short to medium chain length (14–18 carbon) fatty acids and their derivatives with dissociation constants (K_d) in the micromole (μmol) range, in a nonspecific manner (Nagnan-Le Meillour et al., 2000; Jacquin-Joly et al., 2001; Briand et al., 2002; Lartigue et al., 2002; Campanacci et al., 2003). However, two orthopteran proteins, SgreCSP4 and LmigII-10, are able to bind larger ligands, such as the fluorescent reporter 1NPN (Ban et al., 2002, 2003). In contrast, pheromone binding proteins (PBP), one type of insect OBP, also bind fatty acid derivatives with K_d values in the μmol range, but are able to distinguish among different fatty acids (Maibeche-Coisne et al., 1997; Maida et al., 2000; Plettner et al., 2000).

Recent screening-based studies that have detected differential expression of *D. melanogaster os-d*-like genes raise more questions about the function of this family. *DmelpebIII* and *Dmelphk3* were identified as putative targets of the clock transcription factor that regulates circadian rhythms (Claridge-Chang et al., 2001; McDonald and Rosbash, 2001) and *Dmelphk3* as a target of the dorsal transcription

factor involved in embryo and tissue development (Stathopoulos et al., 2002). Additionally, *DmelpebIII* and *Dmelphk3* were found to be immune responsive when challenged with virus and bacteria, respectively; functions ranging from tissue repair to the recognition of invading pathogens were suggested (Sabatier et al., 2003). Similarly, transcription of *Agamir7* increased 6 hr after adult mosquitoes were challenged with bacterial lipopolysaccharide (Oduol et al., 2000). Interestingly, *DmelpebIII* and *Agamir7* are both members of Diptera Class I identified in Figure 4. Finally, *DmelpebIII* was also identified as a candidate gene responsible for a smell impaired mutant phenotype (Anholt and Mackay, 2001). Clearly, further research is required to determine the specific functions of OS-D-like proteins.

We have cloned four new members of the insect *os-d*-like gene family, and identified 11 more from sequence databases, allowing the identification of four new protein similarity classes (Lepidoptera Class 1 and 4, Hymenoptera Class 1, and Diptera/Hymenoptera Class 1) (Figure 4). Orthoptera Class 1 and 2, and Lepidoptera Class 2 and 3, have been described previously (Jacquin-Joly et al., 2001; Picimbon et al., 2001; Ban et al., 2003). Each similarity class was characterized by the retention of, or diversion from, several highly conserved motifs. Although even a single residue substitution can have profound effects on protein function, the conserved motifs must impart some level of common function to members that retain them. The recently solved crystal and NMR structure of MbraCSPA6 from the moth *M. brassicae* (Lartigue et al., 2002; Campanacci et al., 2003; Mosbah et al., 2003) provides an insight into functional constraints that may be contributing to the conservation of some residues.

MbraCSPA6 is a small globular protein composed of six amphiphatic alpha helices that surround an internal hydrophobic pocket; the four conserved Cys residues form two disulphide bonds that create α–α loops (Figure 6, Lartigue et al., 2002; Mosbah et al., 2003). Three amino acid positions (27, 85, and 98, Figure 2) are highly conserved as aromatic residues within the OS-D-like family. In MbraCSPA6, a tyrosine residue located at position 27, and a tryptophan residue at position 98, may act as gates to the hydrophobic pocket (Lartigue et al., 2002; Mosbah et al., 2003) (Figures 2 and 6). Evidence indicates that the tryptophan residue at position 85 of AmelCSP3b faces the binding pocket and may interact with the ligand (Briand et al., 2002). Several residues that are located at either mouth to the hydrophobic pocket of MbraCSPA6 are also conserved within the OS-D family. These include residues at positions 12, 14, and 17 of Figure 2 (N-terminal mouth) and 66/67 and 101 (C-terminal mouth) (Lartigue et al., 2002; Mosbah et al., 2003). Thus, residues involved in ligand binding may be functionally conserved.

Conserved motifs exposed on the surface of the protein could be involved in protein regulation and/or interactions. The N-terminal sequence of MbraCSPA6, where the conserved motif A, YTTKYDN(V/I)(N/D)(L/V), is located, is predicted to form an extended region (although less ordered) when in solution

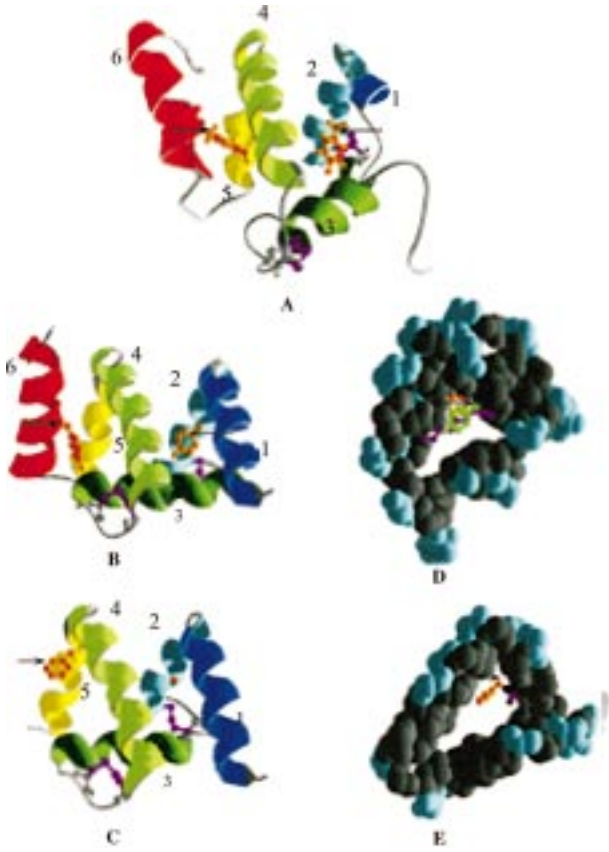

FIG. 6. Ribbon drawings of: A) One of 20 energy minimized NMR structures of MbraCSPA6, Protein Data Bank, PDB (Berman et al., 2000) # 1K19 (Mosbah et al., 2003); B) The crystal structure of MbraCSPA6, PDB # 1KX9 (Lartigue et al., 2002); and C) MsexSAP1 as determined by homology modeling with the crystal structure of MbraCSPA6 (PDB # 1KX9). ?-Helices are numbered 1 through 6 in succession from the N-terminus. The position of the aromatic side chains of residues 27 & 98 are indicated by an arrow (in the case of Figure 6C, the aromatic side chain at position 85 is displayed; position 98 and the 6th ?-helix are missing). D) Cross section view of a space filling model of MbraCSPA6 in complex with 12-bromododecanol (PDB # 1N8V, Lartigue et al., 2002). Residues shaded light grey are predicted to have more than 30% of their surface area exposed (accessible) to the surrounding solvent. The aromatic residues at positions 27 & 98 are indicated by an arrow. E) Cross section view of MsexCSP1, as determined by homology modeling with the crystal structure of MbraCSPA6 (PDB # 1KX9). The images and initial models were created using Deep View/Swiss-PdbViewer 3.7 software (Guex and Peitsch, 1997); models were optimized by Swiss-Model software on the ExPASy molecular biology server (www.us.expasy.org).

(Figure 6A; Mosbah et al., 2003). Motif B, DGKELKXX(I/L)PDAL, spans the third amphiphatic α-helix; within this region, the aspartic acid (D), glutamic acid (E), and lysine (K) residues are exposed at the surface of the protein, whereas the remaining hydrophobic residues contribute to the hydrophobic pocket.

OS-D-like proteins that have diverged from the conserved motifs share less sequence identity with MbraCSPA6; homology modeling, however, predicts that they form similar 3-D structures. For example, MsexSAP1 and Diptera/Hymenoptera Class 1 (Figure 4) lack both putative aromatic gate residues, and are truncated at the C-terminal end (Figure 2). The homology model of MsexSAP1, however, indicates that the general 3-D structure of the protein is maintained, even with the loss of the sixth α-helix (Figure 6C and E). Interestingly, the conserved aromatic residue at position 85 (that faces the internal pocket of AmelASP3C) appears to be able to take a position where it could act as gate, similar to the aromatic group at position 98 of proteins that retain the sixth α-helix (Figure 6B). Thus, the basic 3-D structure of MbraCSPA6 may be conserved within all OS-D-like classes identified herein, including those that have diverged from the conserved motifs. CD spectrum and NMR data from AmelASP3C and SgreCSP4 indicate that they have the same general fold and disulphide bonding pattern (Picone et al., 2001; Briand et al., 2002).

Highly conserved motifs among members representing the orders Diptera, Hymenoptera, and Lepidoptera, as well as the Dictyoptera, Orthoptera, and Phasmatodea, indicate that a common ancestral *os-d*-like gene predated the ancestral Neoptera (Figure 5). Therefore, similar genes should be discovered in other Neopteran orders, such as the Coleoptera and Hemiptera. The degree of diversification that may have occurred prior to the ancestral Neoptera is not clear; many of the OS-D-like similarity classes may have diverged within specific orders, but at least one class appears to have diverged prior to the Diptera/Hymenoptera taxonomic division. While most classes are represented at the family or higher level of taxonomy, Orthoptera Class 1 and 2, characterized by high levels of sequence identity, provide some evidence for more recent diversification; if this is the case, then these genes may be clustered in the genome.

The *D. melanogaster* genome has 51 different OBP genes, but their deduced amino acid sequences share little homology (Graham and Davies, 2002; Hekmat-Scafe et al., 2002; Vogt et al., 2002). A large family of divergent proteins may be an adaptation required for the binding of diverse odorant ligands (Vogt et al., 1999). By comparison, the genomes of *D. melanogaster*, *D. pseudoobscura*, *A. gambiae*, and *A. melifera* have fewer *os-d*-like genes (4, 4, 7, and 6, respectively). Also, it has been noted that OS-D-like proteins from diverse taxonomies share more than 40 to 60% identity (Angeli et al., 1999; Marchese et al., 2000; Nagnan-Le Meillour et al., 2000) as compared to OBPs that share 10 to 38% identity (Vogt et al., 1999). Sequence identities of 40% can be largely accounted for by the conserved A-C motifs described herein (for example, up to 40 conserved residues between

proteins with about 110 total residues). It should be noted that protein classes identified herein that diverge from the conserved A-C motifs share less homology with other family members (Figure 4). For example, the median sequence identity (variable signal peptide removed) of AmelCG2, CfumAY426538, and MsexSAP1 with other family members (outside of their similarity class) is 18% (14–27), 25% (18–30), and 21% (16–27), respectively. Whether OS-D-like proteins have functions similar to that of OBPs remains to be determined. Regardless, it will be interesting to investigate the function of proteins that occur both in sensory (sensillum lymph) as well as nonsensory tissues, and that share amino acid motifs (and presumably functional constraints) that have been conserved across diverse insect orders.

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ORIENTATION AND FEEDING RESPONSES OF THE POLLEN BEETLE, *Meligethes aeneus*, TO CANDYTUFT, *Iberis amara*

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Abstract—The pollen beetle, *Meligethes aeneus*, which is an important pest of oilseed rape, *Brassica napus*, and turnip rape, *B. rapa* var. *campestris*, does not oviposit in all species of the Brassicaceae. The relationship between *M. aeneus* and candytuft, *Iberis amara* (Brassicacae), was investigated as part of chemical ecological studies into the development of control methods employing non-host-derived repellents. In choice and nonchoice feeding tests, *M. aeneus* completely rejected *I. amara*. However, in a field experiment using traps baited with flowering racemes of *I. amara* and *B. napus*, *M. aeneus* was attracted to both species. Gas chromatographic (GC) and GC-electroantennogram (GC-EAG) analyses indicated that the profiles of the floral volatiles of the two species are different. At least 12 compounds among the *I. amara* floral volatiles were detected by the *M. aeneus* antenna, and, of these, hexanoic acid, (*E*)-4,8-dimethyl-1,3,7-nonatriene and α-cedrene were not found among *B. napus* flower volatiles. Since *M. aeneus* is stimulated by floral volatiles to approach *I. amara*, but rejects it near, or at, the plant surface, *I. amara* does not produce repellents that could be used to manipulate *M. aeneus*. However, it may contain feeding deterrent(s) that could be used in "push–pull" control techniques or in the development of resistant brassicaceous crops.

Key Words—*Meligethes aeneus*, pollen beetle, Coleoptera, Nitidulidae, *Iberis amara*, candytuft, *Brassica napus*, oilseed rape, Brassicaceae, orientation, feeding, host plant selection, floral volatiles, GC-EAG.

INTRODUCTION

The bronzed blossom (pollen) beetle, *Meligethes aeneus* (Coleoptera: Nitidulidae), a major pest of some brassicaceous crops, feeds in the spring on

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pollen from several plant families, including the Brassicaceae, but oviposits only in brassicaceous buds (Scherney, 1953; Fritzsche, 1957; Free and Williams, 1978; Nilsson, 1989). *M. aeneus* orients to plants by using both visual and volatile chemical cues (Görnitz, 1953; Nolte, 1959; Taimr et al., 1967), and some of the volatile compounds from the host plant oilseed rape, *Brassica napus*, attract or repel the beetle when released individually into the field (Blight and Smart, 1999; Smart and Blight, 2000, and references therein). However, no specific feeding and oviposition stimulants or deterrents for *M. aeneus* have so far been identified.

M. aeneus oviposits in a number of *Brassica* spp., including yellow and black mustards, *B. juncea* and *B. nigra*, and Abyssinian cabbage, *B. carinata* (Ekbom and Borg, 1996), and it is a particular pest of oilseed rape, *B. napus*, and turnip rape, *B. rapa* var. *campestris* (Bromand, 1990). Some other brassicaceous plants, e.g., white mustard, *Sinapis alba*, and crambe, *Crambe abyssinica*, are acceptable for feeding, but are generally not colonized (Winfield, 1961; Ekbom and Borg, 1996). Winter cress, *Barbarea vulgaris*, and land cress, *B. verna*, have low acceptability for both feeding and oviposition (Börjesdotter, 2000).

Brassicaceous species that are unacceptable, or less acceptable, for feeding and oviposition by pests of brassica crops could be of value in developing alternative methods for the control of these insects. For example, such plant species could be used as repellent intercrops, and any derived repellents or feeding deterrents might be used to manipulate insect populations by using "push–pull" control techniques (Smart et al., 1994). Additionally, various plant breeding techniques including somatic hybridization and genetic engineering might be used to develop resistant brassicaceous crops incorporating attributes from these plants. Therefore, we undertook a study of candytuft, *Iberis amara* (Brassicaceae), within the broader framework of our studies on chemical ecology related to the development of alternative methods of pest control (Pettersson et al., 1994; Smart et al., 1994; Pickett et al., 1995; Guerrero et al., 1997).

No reports of *M. aeneus* feeding on, or colonizing, *I. amara* were found in an extensive literature search. Moreover, there are few reports of *I. amara* as a host for other brassica pests, although the mustard beetle, *Phaedon cochleariae*, and the flea beetle, *Phyllotreta tetrastigma* (Nielsen, 1978a), will feed on candytuft to some extent, and the plant is acceptable to the butterfly, *Pieris napi oleracea*, for oviposition (Huang and Renwick, 1993). However, several other *Phyllotreta* spp. flea beetles, including *P. cruciferae* (Feeny et al., 1970), *P. nemorum* and *P. undulata* (Nielsen, 1978b, 1989), and *P. armoraciae* (Nielsen, 1978b; Nielsen et al., 1979) are deterred from feeding, and the plant is also unacceptable to the weevil, *Ceutorhynchus constrictus* (Nielsen et al., 1989), the cabbage stem flea beetle, *Psylliodes chrysocephala* (Bartlet and Williams, 1991), and the larvae of *Pieris rapae* (Sachdev-Gupta et al., 1993). Oviposition by *P. rapae* is also deterred (Huang and Renwick, 1993).

I. amara contains glucosinolates, nonvolatile thioglycosides that are characteristic of the Brassicaceae (Gmelin, 1963; Ettlinger and Kjaer, 1968), and stimulate feeding by many insects (Städler, 1992). This plant also contains cucurbitacins, oxygenated tetracyclic triterpenes (Curtis and Meade, 1971; Lavie and Glotter, 1971) that may be either deterrents or stimulants of feeding and oviposition. Of the brassica pests that do not feed on *I. amara*, *Phyllotreta nemorum* and *P. undulata* adults, and *Pieris rapae* larvae, are deterred, at least in part, by cucurbitacins E and I, or their glycosides, which are present in *I. amara* foliage (Nielsen et al., 1977; Nielsen, 1978a; Sachdev-Gupta et al., 1993). The same compounds deter oviposition by *P. rapae*, but *P. napi oleracea* is behaviorally insensitive to them (Huang et al., 1993).

It is not known if pests of brassica crops that are unadapted to *I. amara* employ volatile semiochemicals as repellents to avoid initial contact with the plant. The objectives of this work were to discover if *I. amara* is acceptable to *M. aeneus* for feeding, and, if not, whether *M. aeneus* is repelled by volatile inflorescence semiochemicals. The results of laboratory feeding experiments on *I. amara* and *B. napus* anthers, and a field experiment on the orientation of *M. aeneus* to *I. amara* and *B. napus* inflorescences, are described. Some volatile chemicals in *I. amara* racemes, which were detected by the *M. aeneus* antenna, were identified.

METHODS AND MATERIALS

Insects. Adult *M. aeneus* used for feeding tests were collected from the field, the day before use, by sweep-netting *B. napus* racemes. Beetles were sexed by gently squeezing the abdomen so that the genitalia were everted, and then maintained overnight at 18°C, without food, before use. For coupled gas chromatography-electroantennography (GC-EAG) and EAG recording, *M. aeneus* were field-collected immediately before use, and their sex was determined later by dissection.

Plants. Flowering racemes used in field experiments and anthers used in feeding experiments were obtained from wild candytuft, *I. amara* (John Chambers Wild Flower Seeds, Kettering, Northants, U.K.) and *B. napus* cv. Topas, grown in the glasshouse.

Chemicals. Pentane and hexane (both HPLC grade, Fisons) were purified as in Blight (1990). Chemicals ($\geq$98% pure) were obtained from commercial sources (Aldrich Chemical, Avocado Research Chemicals, Fluka Chemie AG, Lancaster Synthesis), except for (*E*)-4,8-dimethyl-1,3,7-nonatriene (96%), which was synthesized (Pattenden and Weedon, 1968).

Feeding Experiments. (i) Choice Tests: *M. aeneus* were allowed to feed on undehisced anthers of *B. napus* and *I. amara*. Sixty beetles were placed individually into stoppered glass tubes (50 $\times$ 17 mm), each of which contained one anther of

B. napus and one of *I. amara* on a damp filter paper disk (13 mm diam). After leaving the beetles to feed for ca. 5 hr at 23°C, the anthers were examined for feeding damage under a microscope. Feeding was evident as a chewed hole in the anther wall and consumption of some, or all, of the pollen within. Beetles that did not feed were discounted.

(ii) Nonchoice Tests: These were as described above for the choice tests, except that an anther of only one species was provided for each beetle. Two sets of 60 replicates were carried out simultaneously, one with *B. napus* anthers and the other with *I. amara.*

Field Experiments. The orientation responses of *M. aeneus* to flowering racemes of *I. amara* and *B. napus* were compared in two field experiments conducted on Rothamsted Farm in 1994 and 1996. Each experiment consisted of four treatments: a control (empty trap); 5 flowering racemes of *I. amara*; 5 flowering racemes of *B. napus*; and a lure of 2-phenylethyl isothiocyanate (NCS) released at 5 mg/d by diffusion from a polyethylene dispenser (Smart and Blight, 1997). The latter compound, a component of *B. napus* volatiles, had been shown in previous field experiments (Blight and Smart, 1999) to attract *M. aeneus* and was used here as a standard. The flowering racemes were watered as required and changed twice weekly.

The trap used was a modified version of a yellow Petri dish water trap (Smart et al., 1997), with a Perspex pot (4 cm diam $\times$ 5 cm deep) glued to the center. The flowering racemes were placed in the pot, which was kept filled with water and covered with a Terylene bag to prevent ingress of insects. A wire frame supported a Petri dish lid that functioned as a rain shield. The pots containing the control and 2-phenylethyl NCS-treated traps were also covered with Terylene bags to present a uniform visual cue. The 2-phenylethyl NCS lure was attached outside the bag to the wire support. The traps, which were filled with detergent solution (0.1% in water), were mounted on poles approximately 1-m above ground level.

A randomized block (Latin square) design was used (Smart et al., 1997; Smart and Blight, 1997), in which traps representing one replicate (i.e., row) of a block were set out in a straight line at 10-m spacing, adjacent to postflowering winter oilseed rape. Captured insects were removed at regular intervals and were identified and counted in the laboratory. A block was rerandomized when at least 20 beetles had been captured in a replicate. Total trap catch data were transformed by $\log_{10}(x + 1)$, and analyses of variance (ANOVA) were performed. Transformed treatment means were compared at $P = 0.05$, using Duncan's multiple range test (Duncan, 1955). Means were then transformed back, and these are given in the Results.

Plant Volatiles. Volatiles were collected from flowering shoots of *I. amara* using a dynamic headspace (air entrainment) technique described previously (Blight, 1990). Cut flowering stems (without leaves) were placed in distilled water in conical flasks (3 $\times$ 50 ml) in a glass culture vessel (5 l). Volatiles were drawn

from the vessel, by using purified air, onto a tube of the adsorbent Porapak Q (1.5 g). At the conclusion of the air entrainment, which lasted 5 d, volatiles were eluted from the Porapak with pentane, and the extract was concentrated by fractional distillation of the solvent using a column of glass helices. Final concentration was done under nitrogen and the extract (200 μl) was stored at -20°C.

Gas Chromatography. Air-entrained volatiles were separated on a 50 m $\times$ 0.32 mm i.d. methyl silicone bonded-phase fused silica capillary column (HP-1) fitted in an Hewlett Packard 5890 gas chromatograph equipped with a split/splitless injector and a flame ionization detector (FID). The carrier gas was hydrogen, and the oven temperature was maintained at 40°C for 5 min and then programmed at 5°/min to 150°C, then at 10°/min to 250°C. Coinjections with reference samples were made under the same conditions.

Gas Chromatography-Mass Spectrometry (GC-MS). The capillary column (50 m $\times$ 0.32 mm i.d. HP-1) was directly coupled to the MS and integrated data system (70–250 VG Analytical). Ionization was by electron impact at 70 eV, 230°C. The GC was maintained at 30°C for 5 min and then programmed at 5°/min to 180°C and then held isothermally. Identifications were made by comparison of the mass spectral data with those of authentic samples and confirmed by peak enhancement when the extracts of volatiles were coinjected with authentic compounds using GC, as above.

Electrophysiology. EAG recordings were made using Ag–AgCl glass electrodes filled with saline solution (composition as in Maddrell, 1969, but without glucose). Antennae were excised and suspended between the two electrodes. The signals generated by the antenna were passed through a high impedance amplifier (Syntech UN-06, Hilversum, the Netherlands), and data storage and processing were carried out with a PC-based interface and customized software package (Syntech). The system for stimulus delivery employed a filter paper strip in a disposable Pasteur pipet cartridge. Samples of test solutions (1 mg/ml in hexane, 10 μl used) were applied to filter paper strips, and the solvent was allowed to evaporate for 30 sec before the paper strip was placed into the cartridge. A 2 sec stimulus (1 l/min) was delivered into a purified airstream that flowed continuously over the preparation. Results were expressed as a percentage of the response to a standard application of (*Z*)-3-hexen-1-ol at the same concentration, and analyzed for significant differences by Student's *t* test. Replicates for each compound comprised preparations from five individual insects.

Coupled GC-EAG recordings were made using the system described previously (Wadhams, 1990). Separation of the entrained volatiles was achieved on an AI 93 gas chromatograph equipped with a cold on-column injector and a FID. The carrier gas was hydrogen, and the column (50 m $\times$ 0.32 mm i.d. HP-1) was maintained at 40°C for 1 min and then programmed at 5°/min to 100°C and then at 10°/min to 250°C. The outputs from the EAG amplifier and FID were monitored simultaneously and analyzed using Syntech software (Syntech, The Netherlands).

TABLE 1. FEEDING OF *M. aeneus* ON ANTHERS OF *B. napus* AND *I. amara*

Test	*M. aeneus* Sex	*N*	Number of weevils feeding *B. napus*	*I. amara*
Choice	♂	30[a]	18	0
	♀	30[b]	19	0
Nonchoice	♂	30	19	—
	♀	30	16	—
	♀	30	—	0
	♂	30	—	0

[a] 12 males did not feed and were discarded.
[b] 11 females did not feed and were discarded.

RESULTS

Feeding Experiments. There were no marked differences in the feeding behavior of male and female *M. aeneus.* In the choice tests (Table 1), 100% of the beetles fed on *B. napus*. In the simultaneous nonchoice tests, more than 50% of each sex fed on *B. napus*, but *I. amara* was completely untouched by both sexes with no trial feeding.

Field Experiments. The individual analyses of the two field experiments (Table 2) indicated that *M. aeneus* was attracted to the flowering racemes of both *I. amara* and *B. napus*, and that there was no significant difference between these treatments. In experiment 1, the *B. napus* racemes, and in experiment 2, the *I.*

TABLE 2. RESPONSE OF *M. aeneus* TO YELLOW WATER TRAPS BAITED WITH FLOWERING RACEMES OF *B. napus* AND *I. amara*

Treatment	*M. aeneus* caught per replicate (mean)[a] Experiment 1[b]	Experiment 2[c]	Experiment 1 + Experiment 2
Unbaited	4.5 b	3.3 b	3.8 c
I. amara racemes	21.3 ab	8.8 a	13.8 b
B. napus racemes	30.0 a	10.3 a	17.7 ab
2-Phenylethyl NCS[d]	92.0 a	12.5 a	34.4 a

[a] Within each column, means followed by different letters are significantly different (Duncan's multiple range test, $P = 0.05$). Means are back-transformed.
[b] 766 *M. aeneus* were caught in four replicates, August 9–16, 1994. ANOVA gave for treatment effect: $F = 8.2; df = 3, 6; P = 0.02$. SE of a treatment mean was 0.18.
[c] 158 *M. aeneus* were caught in four replicates, June 14–July 1, 1996. ANOVA gave for treatment effect: $F = 6.1; df = 3, 6; P = 0.03$. SE of a treatment mean was 0.09.
[d] 2-Phenylethyl isothiocyanate (NCS) was released at 5 mg/d.

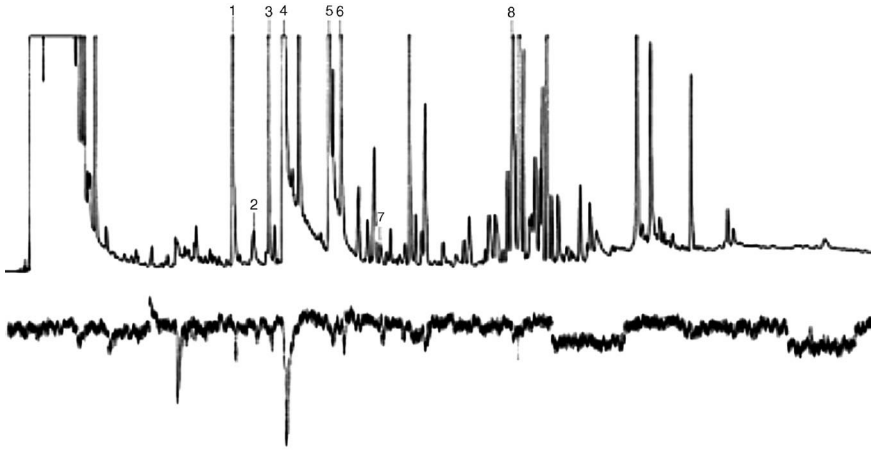

FIG. 1. Coupled gas chromatography-electroantennogram recording (GC-EAG) from *Meligethes aeneus.* Upper trace: GC of volatiles from flowering shoots of *Iberis amara;* Lower trace: EAG responses: 1, benzaldehyde; 2, hexanoic acid; 3, (*Z*)-3-hexenyl acetate; 4, phenylacetaldehyde; 5, 2-phenylethanol; 6, (*E*)-4,8-dimethyl-1,3,7-nonatriene; 7, methyl salicylate; 8, α-cedrene.

amara and *B. napus* racemes, were all significantly more attractive than the unbaited trap. The trap with *I. amara* racemes in experiment 1 caught more *M. aeneus* than the unbaited trap, but this treatment just failed to be significantly different from the unbaited trap (Duncan's multiple range test, $P = 0.05$). Inspection of the raw data showed that this was due to an abnormally large catch in the unbaited trap in one of the four replicates. However, combined analysis of all eight replicates from experiments 1 and 2 clearly indicated that, at $P = 0.05$, the *I. amara* and *B. napus* treatments both caught significantly more *M. aeneus* than the unbaited trap. Thus, as well as orienting to *B. napus* in the field (Taimr et al., 1967), *M. aeneus* also orients to *I. amara.*

Analysis of Volatiles from I. amara *Inflorescences.* The profile of volatiles (Figure 1) was different from that found for *B. napus* (Blight et al., 1995). Coupled GC-EAG (Figure 1) indicated that the *M. aeneus* antenna detected at least 12 compounds in the *I. amara* extract of volatiles. Of the 12, eight were tentatively identified by coupled GC-MS, and the identifications were confirmed by GC coinjection with reference samples (Table 3). Three of these eight compounds, hexanoic acid, (*E*)-4,8-dimethyl-1,3,7-nonatriene, and α-cedrene, are not present in *B. napus* (Blight et al., 1995). Electrophysiological activity, at the 10^{-5} g level, of seven of the eight compounds was confirmed by EAG recording using authentic samples (Table 3), but at this concentration the sesquiterpene α-cedrene was inactive.

TABLE 3. EAG RESPONSES OF FEMALE *M. aeneus* TO VOLATILES RELEASED BY *I. amara*, EXPRESSED AS A PERCENTAGE OF RESPONSE TO (*Z*)-3-HEXEN-1-OL

Peak no.	Compound (10^{-5} g)	Response[a] ($\pm$SE)
1	Benzaldehyde	24 ± 7.9*
2	Hexanoic acid	66 ± 6.5***
3	(*Z*)-3-Hexenyl acetate	60 ± 2.1***
4	Phenylacetaldehyde	124 ± 38.6*
5	2-Phenylethanol	86 ± 12.1***
6	(*E*)-4,8-Dimethyl-1,3,7-nonatriene	44 ± 9.9**
7	Methyl salicylate	97 ± 14.6***
8	α-Cedrene	13 ± 5.1 NS

Note. Response to standard application of 10^{-5} g (*Z*)-3-hexen-1-ol was 0.43 mV $\pm$ 0.1 (compounds 1, 4–8) and 0.28 ± 0.07 (compounds 2, 3). *, **, ***: significantly different from control at $P = 0.05$, 0.01, 0.001, respectively. NS = not significantly different from control at $P = 0.05$ (control = hexane, 10 μl).

[a] Replicates for each compound comprised preparations from five individual insects.

DISCUSSION

The absence of feeding by *M. aeneus* on *I. amara* suggests either that the plant lacks a feeding stimulant or that it contains a feeding deterrent. There is a possibility that induced feeding preference influenced the choice of *B. napus* rather than *I. amara* for feeding in the choice tests, because the insects had been collected from *B. napus* (Hsiao, 1985). However, the results of the nonchoice tests indicate that this is unlikely. There were no obvious physical barriers to feeding on *I. amara*, and rejection was not due to satiation since a high proportion of the insects fed on *B. napus.*

The putative feeding stimulants sinigrin (2-propenyl glucosinolate), 3-methylthiopropylglucosinolate, and glucoiberin (3-methylsulfinylpropylglucosinolate) have all been detected in seeds and foliage of *I. amara* (Kjaer, 1960; Cole, 1976; Huang et al., 1993), and there is indirect evidence for their presence in inflorescences (Dalgaard et al., 1977). Using GC-MS, we searched for volatile catabolites of these compounds among the flower volatiles, but did not find any. Failure to detect them does not necessarily mean the parent glucosinolates were absent because the flowering racemes were intact, and the catabolites are normally produced when the plant is damaged. However, the effects of any feeding stimulants that may be present are outweighed by the presence of feeding deterrents in the anthers. These may be cucurbitacins (see Introduction) and/or other compounds. Their identification would be of interest, and relevant to work on noninsecticidal methods for control of *M. aeneus*, e.g., as a

contribution to the "push" element of the push–pull control technique (Smart et al., 1994).

The attraction of *M. aeneus* to *I. amara* observed in the field experiment was mediated solely by volatile compounds from the inflorescences and not by any visual cues, because all the treatments were enclosed in opaque bags. However, both visual and olfactory cues are involved in the normal orientation of *M. aeneus* to *B. napus* in the field (Blight and Smart, 1999, and references therein), and both are also likely to be involved in orientation to *I. amara*.

Seven of the eight compounds identified among the *I. amara* inflorescence volatiles, and found by coupled GC-EAG to stimulate the antenna (Figure 1), also elicited EAG responses when tested alone at 10^{-5} g. However, α-cedrene did not, and, thus, it is possible that the observed GC-EAG response may have been elicited by another compound, coeluting with α-cedrene, but present in too small an amount to be identified by GC-MS. All eight of the compounds (Table 3) had been previously tested alone in the field (Smart and Blight, 2000; Smart, unpublished). Six, namely benzaldehyde, (*Z*)-3-hexenyl acetate, phenylacetaldehyde, 2-phenylethanol, (*E*)-4,8-dimethyl-1,3,7-nonatriene, and methyl salicylate, were attractive to *M. aeneus* when released at rates of 1–26 mg/d (Smart and Blight, 2000). The other two, hexanoic acid and α-cedrene, were inactive when tested at similar release rates (Smart, unpublished), but nevertheless they may contribute to the complex floral bouquet that attracts the beetles to *I. amara*.

Taken together, the results suggest that *M. aeneus* is stimulated by volatiles to approach *I. amara*, but rejects it during sampling of the plant surface by using contact chemoreceptors on the antennae, tarsi, and labia (Städler, 1992). A similar relationship may also exist between *M. aeneus* and *Barbarea* spp. because *M. aeneus* is attracted to the inflorescences in the field, but apparently does not damage the buds (Merker and Nilsson, 1995). There are increasing numbers of reports of plant volatile chemicals that repel insects. Repellency, or masking of odor, has been demonstrated in laboratory bioassays (Liu et al., 1988; Nottingham et al., 1991; Hardie et al., 1994), and field studies have confirmed that plant volatile chemicals may cause rejection of the plant. Thus, unsuitable host trees are detected by the pine shoot beetle, *Tomicus piniperda*, while in flight (Byers et al., 1989); methyl salicylate decreases colonization of cereals by the aphids *Rhopalosiphum padi*, *Sitobion avenae*, and *Metopolophium dirhodum* (Pettersson et al., 1994); benzyl alcohol deters attacks of the pine shoot beetle *T. destruens* on *Pinus pinea (*Guerrero et al., 1997); and the floral scent of *Osmanthus fragrans* deters foraging and repels *Pieris rapae* (Ômura et al., 2000). If *I. amara* volatiles had proven to be repellent to *M. aeneus*, their use as the "push" component of a stimulodeterrent diversionary control method would have been worth investigation (Smart et al., 1994).

Although, in many cases, host plant volatiles appear to attract insects directly to the plant, it has also been suggested that insects are arrested in the vicinity of plants, where, as a result of visual cues, indiscriminate landing occurs, followed

by acceptance or rejection (see Finch and Collier, 2000, and citations therein). There is some evidence for this, e.g., it was concluded that host plant finding in the Colorado potato beetle is a "chance event" (Jermy et al., 1988), even though olfactory orientation of the beetle to potato volatiles has been demonstrated in laboratory experiments (Visser, 1988, and citations therein), and in field studies (Dickens and Alford, 2002).

There is increasing evidence that associative learning occurs commonly with insects (Papaj and Lewis, 1993; Bernays and Chapman, 1994) and this has important implications for insect–plant relationships. Food aversion learning has been demonstrated with several herbivorous insects (Bernays, 1993), and avoidance learning of odor blends by parasitoids may occur (Turlings et al., 1993). One may speculate that, if *M. aeneus* is in regular contact with *I. amara*, it may learn to associate the flower odor with the presence of feeding and oviposition deterrents, and the cocktail of flower volatiles would then become repellent. However, if the deterrents in *I. amara* were giving a false signal of plant unacceptability, there could be a shift in behavior and *M. aeneus* would then colonize the plant. Extension of host plant range has occurred with other brassica specialist insects, viz. *Pieris napi oleracea* on *I. amara* (Huang and Renwick, 1993), and *Phyllotreta nemorum* on *Barbarea vulgaris* (Brassicaceae) (Nielsen, 1996).

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GLANDULAR TRICHOME EXTRACTS FROM *Medicago sativa* DETER SETTLING BY THE POTATO LEAFHOPPER *Empoasca fabae*

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Abstract—Extracts of glandular trichomes from *Medicago sativa* were tested for their ability to disrupt the settling behavior of the potato leafhopper, *Empoasca fabae*. Erect and procumbent glandular trichomes were mechanically isolated from stem sections of resistant genotype "G98A," and nonglandular trichomes were collected from susceptible cultivar "Ranger." Isolated trichomes were extracted with chloroform, acetone, and ethanol, and the resulting crude extracts were applied to the surface of a sachet containing an artificial diet. Leafhoppers were offered a two-way choice between crude trichome extracts from G98A and Ranger. All three of G98A solvent extracts caused various degrees of diet rejection, resulting in the crude Ranger trichome extracts being preferred over G98A extracts. Overall, the fewest leafhoppers settled on the ethanolic extracts. Additional bioassays documented a dose response associated with G98A ethanolic extracts when compared with Ranger trichome extracts and a solvent control. No difference in preference behavior was detected between Ranger trichome extracts and a solvent control. Gas chromatography–mass

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spectrometry analysis revealed a homologous series of nonvolatile fatty acid amides $C_nH_{2n+1}NO$ ($n = 19$–23) unique to G98A glandular trichome extracts.

Key Words—*Empoasca fabae*, potato leafhopper, Homoptera, Cicadellidae, *Medicago sativa* L., alfalfa, glandular trichomes, host-selection, deterrents, fatty acids.

INTRODUCTION

Commercial varieties of glandular-haired (GH) alfalfa, *Medicago sativa* L., are being marketed for control of the potato leafhopper, *Empoasca fabae* (Harris). *Medicago* spp. and genotypes possess two distinct types of glandular trichomes, erect and procumbent, both of which sequester and secrete an exudate (Kreitner and Sorensen, 1979a,b; Ranger and Hower, 2001a; Shockley and Backus, 2002). The glandular secretion can adhere to the leafhopper's cuticle (Ranger and Hower, 2001a). However, physical entrapment of the potato leafhopper by the glandular trichome exudate is not the primary causative factor of resistance (Elden and McCaslin, 1997; Ranger and Hower, 2001a,b; Shockley et al., 2002).

Studies have indicated that resistance of GH *M. sativa* to the potato leafhopper is based more on chemical, rather than mechanical, mechanisms. For instance, decreased nymphal and adult survival occurred on several GH *M. sativa* genotypes (Hogg and McCaslin, 1994; Elden and McCaslin, 1997; Ranger and Hower, 2001b; Shockley et al., 2002). Nymphs also exhibited reduced growth rates (Hogg and McCaslin, 1994). However, mechanical removal of the glandular trichomes from the *M. sativa* genotype FGplh13 (Forage Genetics, Inc.) eliminates the resistance; leafhopper mortality and development does not differ significantly from that on a susceptible, nonglandular trichome cultivar (Ranger and Hower, 2001b).

The presence of glandular trichomes also affects leafhoppers' host-selection behavior (Hogg and McCaslin, 1994; Ranger and Hower, 2002; Shockley and Backus, 2002). First and third instars changed their settling location more frequently on the GH genotype FGplh13 compared to a susceptible control (Ranger and Hower, 2002). In addition, time–course analyses of settling by adult potato leafhoppers show that susceptible nonglandular stems are preferred over stems of the GH genotype FGplh13 (Ranger and Hower, 2002). Stems of FGplh13 from which the glandular trichomes were mechanically removed were also preferred over FGplh13 stems with intact gland heads.

The aforementioned studies indicate that *M. sativa* glandular trichomes play a role in conferring resistance to potato leafhoppers, apparently due to the localization of biologically active compounds within these secretory structures. Elden and McCaslin (1997) proposed the glandular trichomes may be producing toxic or repellent volatile compounds, or a nonvolatile toxin that leafhoppers must come in contact for resistance to occur. However, such a purported chemical basis for leafhopper resistance has not been studied. In particular, documentation of the

effects of *M. sativa* glandular trichome extracts on leafhopper settling behavior is lacking, as is knowledge of the chemical composition of the glandular secretions.

Here, we report for the first time that crude glandular trichome extracts from GH *M. sativa* G98A (Cal/West Seeds), when directly applied to the surface of an artificial diet sachet, deter settling by the potato leafhopper. Chromatographic and mass spectrometric analyses indicate that nonvolatile lipophilic amides are unique to the glandular trichomes of the resistant *M. sativa* genotype G98A and may be responsible for deterring leafhopper settling.

METHODS AND MATERIALS

Insects and Plants. A colony of potato leafhoppers was maintained according to Hunter and Backus (1989). Insects were reared on greenhouse-grown fava beans *Vicia faba* (cv. "Windsor") in an environmental growth chamber (temperature: 25 ± 2°C; photoperiod: 16:8 hr L:D). Cuttings (ramets) of resistant GH *M. sativa* genotype G98A, and less resistant GH genotype G98C, were provided by Cal/West Seeds (West Salem, WI), along with susceptible nonglandular-haired cultivar Ranger. Plants were vegetatively propagated and grown under greenhouse conditions. Metal halide lamps (photoperiod: 16:8 hr L:D) were used to supplement the natural lighting. Experimental plants were about 4 mo old and harvested three times prior to use.

Trichome Isolation and Extraction. Trichomes were isolated from stem sections of G98A and Ranger according to a modified protocol of Yerger et al. (1992). Entire stems were harvested from 11:00 A.M. to 3:00 P.M. and immediately cut into 2–5 cm sections. About 1–2 g of the stem sections were transferred into a test tube and lowered into a Dewar flask containing liquid N_2. After submersion, the test tube was raised out of the solution, and the liquid N_2 was allowed to evaporate. The test tube was vortexed for 3–5 sec, resulting in the free trichomes adhering to the test tube walls.

Trichomes were isolated from 25 g G98A and Ranger stems for analytical purposes and soaked in methylene chloride with 10 g Na_2SO_4. After 24 hr, samples were filtered through glass fiber circles (G6, Fisher Scientific, Pittsburgh, PA) and concentrated to dryness with a Kuderna–Danish evaporative concentrator (Kontes Glass Company, Vineland, NJ). Residues were redissolved in methylene chloride for analysis (see Analysis of Trichome Extracts).

Trichomes were isolated from 75 g of G98A and Ranger stems to obtain extracts for bioassay purposes. Trichomes were initially extracted (on separate occasions) with chloroform, acetone, and ethanol. Bioassays were conducted to determine which solvent was most effective in extracting biologically active compounds. All subsequent trichome extracts prepared for bioassay purposes were made using ethanol. Trichomes were soaked for 24 hr, filtered, and evaporated

under reduced pressure by using a rotary evaporator. Residues were redissolved in acetone and concentrated to 2 ml under a gentle stream of N_2. Weight of G98A and Ranger residues obtained using chloroform, acetone, and ethanol were 8.0 and 4.8 mg, 4.5 and 1.5 mg, and 10.2 and 4.4 mg, respectively. These stock solutions were stored at -8°C until use.

Glandular trichomes were also extracted individually using the technique of Walters et al. (1989). A finely drawn Pasteur pipette and capillary action were used to draw up a small volume of methylene chloride, and an individual erect glandular trichome was then inserted into the tip of the pipette and extracted for 20 sec. Collections from 500 erect glandular trichomes each from the resistant G98A and less-resistant G98C were blown into a chilled collection vial and allowed to evaporate slowly to dryness in a sterile fume hood. Extracts were redissolved in 20 μl methylene chloride for analytical analysis.

Bioassay of Trichome Extracts. Two-way choice tests were used to test deterrency of crude trichome extracts. Prior to each bioassay, <5-d-old adult females were selected from the laboratory colony and transferred to a cage containing two mature Ranger plants for a 24-hr period of conditioning. Insects were transferred to a sachet containing an artificial diet (adapted from Habibi et al., 1993) for an additional 24 hr of acclimation. The artificial diet consisted of an aqueous solution of 5% (wt:vol) sucrose and 4% (wt:vol) low-melt agarose (Sea Plaque Agarose, FMC Inc., Rockland, ME). The acclimation sachet was prepared by wrapping tape around the bottom portion of an inverted petri dish (diam. 8.5 cm). Sterile artificial diet was poured into the petri dish and allowed to solidify. Tape was removed, leaving a disk of artificial diet. Parafilm® was stretched over the solidified diet and trimmed to within the edges of the diet. The sachet was placed in the bottom of the petri dish with the Parafilm® surface exposed, and a petri dish lid was used to contain the leafhoppers.

Deterrency was tested by using a second, smaller sachet, based on a design by Habibi et al. (1993). Smaller sachets were prepared by first pipetting 100 μl artificial diet into a plastic gasket (1×1 cm^2) positioned on a glass microscope cover slip. Parafilm was stretched over the diet and surrounding gasket and trimmed back to within the edges of the gasket. Bioassay sachets were prepared 24 hr before use and were stored at 1°C for 24 hr.

Trichome extracts were tested at concentrations representing 7.5, 3.7, 1.8, 0.25, and 0.125% of the initial 2 ml stock solution (see Trichome Isolation and Extraction). Therefore, the same proportions of sample volume from extracts were applied to the sachets for each experiment. This resulted in G98A and Ranger extracts being compared at 700 and 310 μg/cm^2; 350 and 155 μg/cm^2; 175 and 75 μg/cm^2; 25 and 10 μg/cm^2; and 10 and 5 μg/cm^2, respectively. Control samples were prepared by applying the corresponding volume of acetone to the sachet surface. Extracts applied to the sachet surface were allowed to air dry prior to use.

Sachets treated with extracts were placed into a chamber consisting of a clear upright plastic tube (7.0 cm diam, and 3.5 cm high) sealed at both ends with petri dish lids. Prior to sealing the arena, sachets were placed at opposing regions within the cage. Acclimated leafhoppers (10 per arena) were aspirated into each arena. Arenas were arranged in a completely randomized design in an aluminum tray, and water was added to prevent desiccation. Trays were placed under constant fluorescent light. After 60 min of acclimation, the number of insects settling/feeding on a particular diet surface was recorded at 15 min intervals over 350 min.

Leafhoppers settling on each sachet were converted into proportions and arcsine square root-transformed. Data were analyzed with the SAS general linear model (GLM) procedure (SAS Institute, 1985) by using a repeated measures split plot analysis of variance (ANOVA). Treatment was used as the main plot effect in the linear statistical model for comparisons of settling. The subplot contained the effects of time and Treatment $\times$ Time. The comparison of leafhopper settling on G98A for each of the three solvents used the main plot effects of solvent, treatment, and Solvent $\times$ Treatment in the linear statistical model. The subplot contained the effect of time and all possible interactions with the main plot effects. Differences among means for ANOVAs were compared with least significant difference (LSD procedure), Statistical Analysis System (SAS Institute, 1985).

Analysis of Trichome Extracts. Methylene chloride extracts of trichomes from 25 g G98A and Ranger stems were analyzed with an Hewlett-Packard 5890 gas chromatograph equipped with a mass spectral detector operating in electron impact (EI) mode (70 eV). A 15 m Restek Rtx-1 column (internal diam 0.25 mm, and film thickness 0.3 μm) was used. The injector port was held at 250°C, and the oven was programmed from 100 to 320°C at 10°C/min and held at 320°C for 5 min. In addition to EI mass spectrometry, fast atom bombardment (FAB) mass spectrometry was conducted with a JEOL MStation JMS 700 with a double focusing MS operating in positive mode. The particle beam was xenon gas, and the matrix was meta-nitrobenzyl alcohol.

To assess further the major components of the erect glandular trichomes on G98A, extracts obtained from the individual trichome extraction procedure (see Trichome Isolation and Extraction) were analyzed by gas chromatography (GC). Five microliters of extracts from 500 individual glandular trichomes were analyzed on an Hewlet-Packard 5890A GC equipped with a flame-ionization detector (FID) and a Restek Rtx-1 column. Injector and detector temperatures were set at 250°C, and the oven was programmed from an initial temperature of 200°C for 2 min, a ramp of 10°C/min to 250°C, and held at 250°C for 5 min. Retention times of compounds from the individually extracted trichomes were compared to retention times from the isolated trichome technique.

RESULTS

Solvent Efficacy. Time–course analysis revealed glandular trichome extracts from *M. sativa* G98A deterred leafhopper settling when applied to the surface of an artificial diet sachet (Figure 1A–C). Deterrency associated with chloroform,

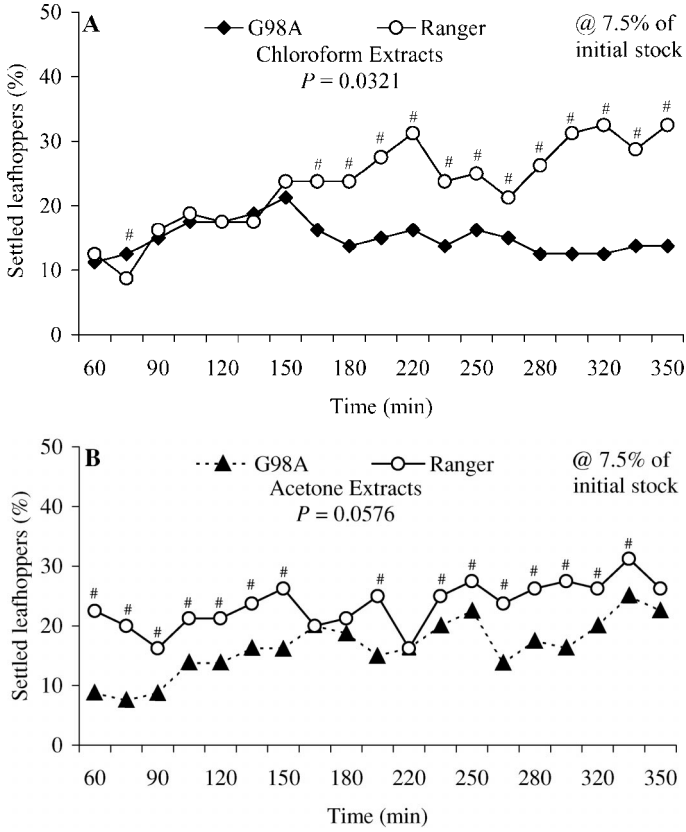

FIG. 1. Comparison of the efficacy of (A) chloroform, (B) acetone, and (C) ethanol to extract biologically active compounds from the trichomes of the glandular-haired *M. sativa* genotype G98A. Leafhopper preference was compared between crude trichome extracts from G98A and the nonglandular cultivar Ranger. Percentage of leafhoppers settling on G98A chloroform, acetone, and ethanol extracts was also consolidated and presented in (D). Extracts were applied to the surface of an artificial diet sachet at a concentration of 7.5% of the initial stock solution (see Methods and Materials for details). Any difference between the percentage of settled leafhoppers greater than an LSD value of 3.6, 3.8, and 6.5% for chloroform, acetone, and ethanol, respectively, are significantly different ($P < 0.05$) and are distinguished by #. An LSD value of 2.4% was used to compare settling on G98A extracts (D). $N = 8$ for each solvent.

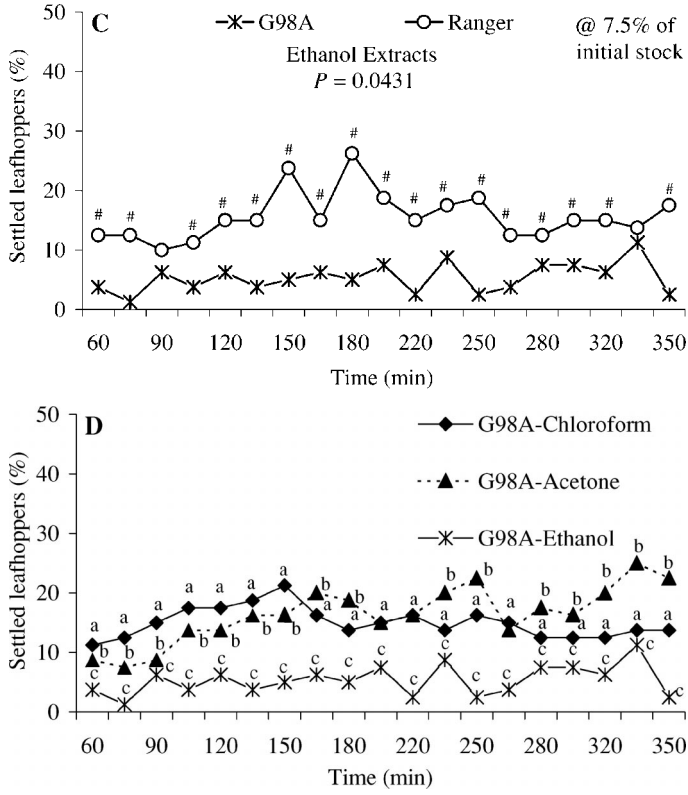

FIG. 1. Continued.

acetone, and ethanol G98A extracts varied. G98A extracts prepared using chloroform and ethanol provided significant degrees of deterrency to leafhopper settling ($F = 7.12$; $df = 1$; $P = 0.032$ for chloroform and $F = 6.08$; $df = 1$; $P = 0.043$ for ethanol). A significant Treatment × Time interaction was also associated with the chloroform extracts ($F = 2.18$; $df = 20$; $P = 0.008$; Figure 1A), but not for the acetone or ethanolic extracts (Figure 1C). Deterrency of G98A acetone extracts was marginally significant ($F = 5.15$; $df = 1$; $P = 0.058$; Figure 1B).

Results of settling tests showed that the fewest leafhoppers settled on the G98A ethanol extracts (Figure 1D). The percentage of leafhoppers preferring G98A ethanolic extracts differed from those preferring G98A chloroform and acetone extracts ($F = 17.34$; $df = 20$; $P < 0.001$). Therefore, ethanol apparently extracted the largest quantity of biologically active compound(s), and so was used to prepare trichome extracts for all subsequent bioassays.

Dose Response of Ethanolic Extracts. The percentage of leafhoppers settling on crude G98A trichome extracts increased with a decrease in sample concentration.

When ethanolic trichome extracts from G98A and Ranger were tested at 3.7% stock solution, or 350 μg/cm^2 for G98A and 155 μg/cm^2 for Ranger, fewer insects settled on the G98A trichome extracts ($F = 27.41$; $df = 1$; $P = 0.001$; Figure 2A). Sachets treated with a solvent control were also preferred over those treated with

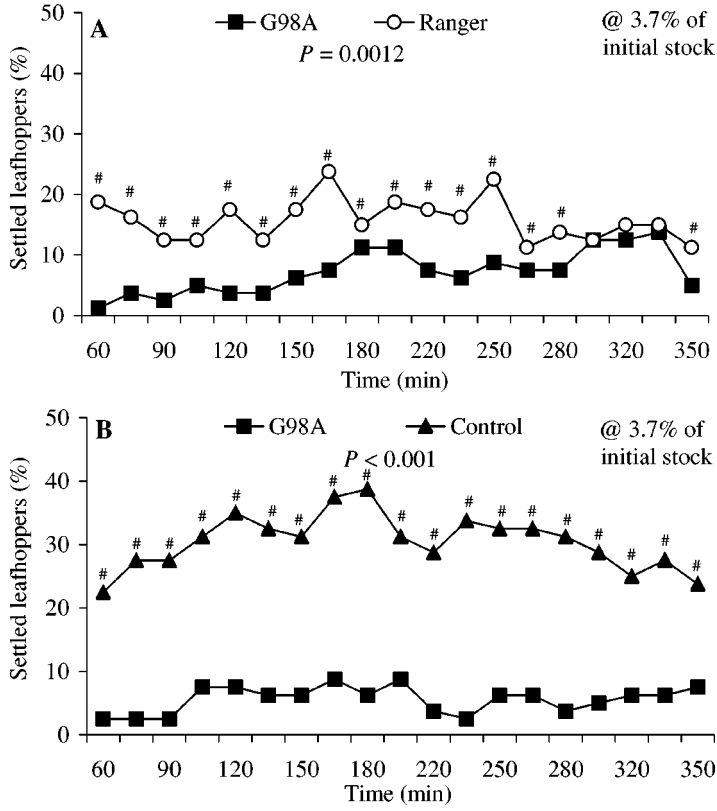

FIG. 2. Preference bioassay of the potato leafhopper for settling on crude ethanolic trichome extracts from resistant glandular-haired genotype G98A and susceptible nonglandular-haired cultivar Ranger. G98A extracts and Ranger extracts were also compared against a solvent control. In A–C, extracts were applied to the surface of an artificial diet sachet at a concentration of 3.7% of the initial stock solution, which represented 350 μg/cm^2 for G98A and 155 μg/cm^2 for Ranger. In D–F, extracts were applied to the surface of an artificial diet sachet at a concentration of 1.8% of the initial stock solution, which represented 175 μg/cm^2 for G98A and 75 μg/cm^2 for Ranger. Extracts were also tested at 0.25 and 0.125% of the stock solution, but data are not shown (see Methods and Materials and Results for details). Any difference between the percentage of settled leafhoppers greater than an LSD value of 3.6% for (A), 3.8% for (B), 3.6% for (D), and 4.3% for (E) are significantly different ($P < 0.05$) and are distinguished by #. $N = 8$ for each comparison.

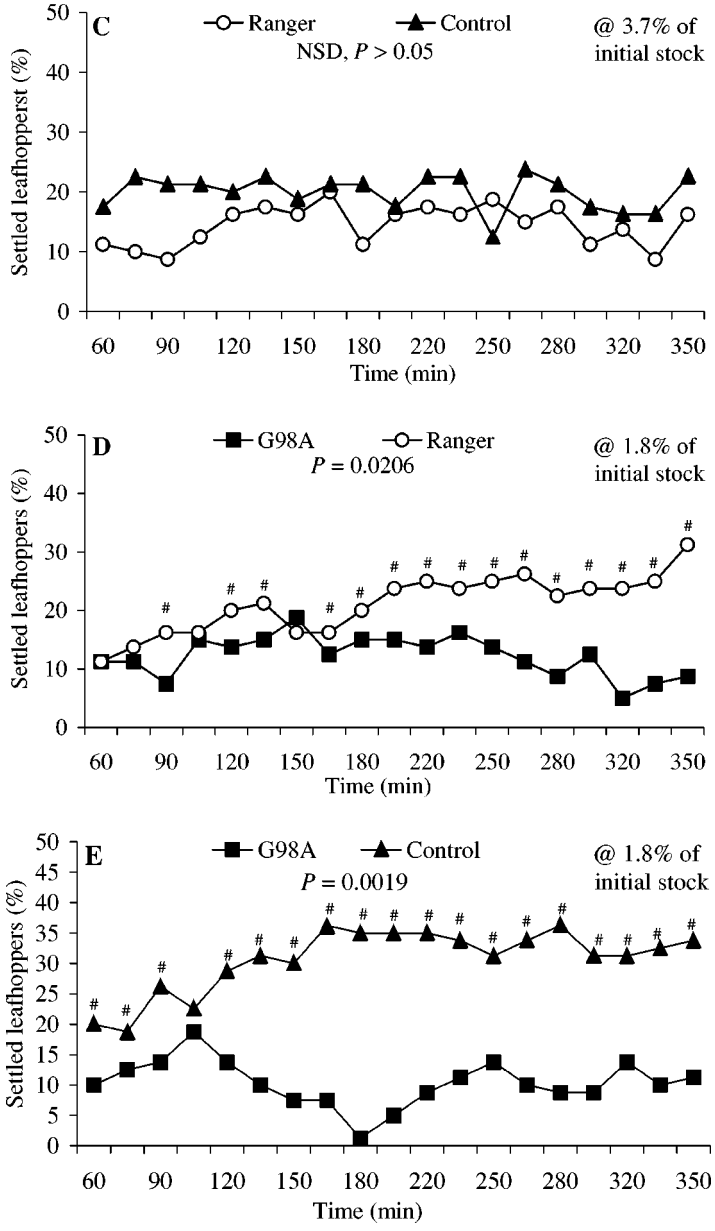

FIG. 2. Continued.

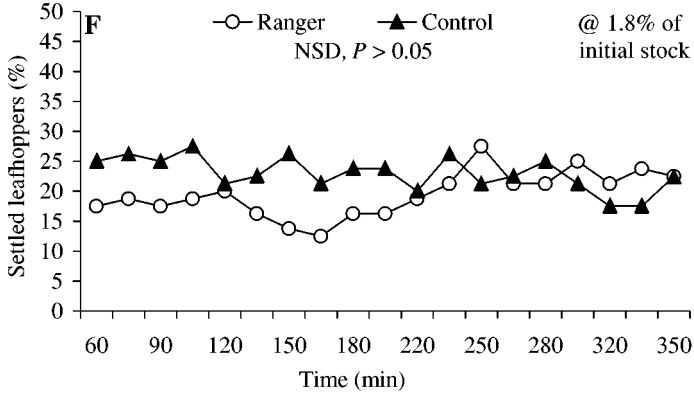

FIG. 2. Continued.

G98A trichome extracts ($F = 103.47$; $df = 1$; $P < 0.001$; Figure 2B). No significant difference was detected among sachets treated with Ranger trichome extracts and solvent control ($P > 0.05$; Figure 2C).

Similar preference patterns were observed when ethanolic G98A and Ranger trichome extracts were tested at a lower concentration of 1.8% stock solution, or 175 μg/cm^2 for G98A and 75 μg/cm^2 for Ranger. Fewer leafhoppers settled on G98A trichome extracts compared to the Ranger trichome extracts ($F = 7.55$; $df = 1$; $P = 0.021$; Figure 2D). A significant Treatment $\times$ Time interaction was detected for this comparison ($F = 2.23$; $df = 20$; $P = 0.002$). Sachets treated with solvent control were also preferred over G98A trichome extracts ($F = 22.65$; $df = 1$; $P = 0.002$), and a significant Time $\times$ Treatment interaction was present ($F = 2.79$; $df = 20$; $P < 0.001$; Figure 2E). In contrast, there was no significant ($P > 0.05$) difference in leafhopper settling behavior among the Ranger extracts and solvent control (Figure 2F).

At 0.25% of the initial stock, or 25 μg/cm^2 for G98A and 10 μg/cm^2 for Ranger, leafhopper settling on G98A and Ranger trichome extracts was not significantly different ($P > 0.05$; data not shown). The solvent control was not preferred over G98A trichome extracts ($F = 4.09$; $df = 1$; $P = 0.074$; data not shown). There was also no significant difference in preference behavior among Ranger trichome extracts and the solvent control ($P > 0.05$; data not shown). Similarly, when G98A and Ranger trichome extracts were compared against one another and also a solvent control at 0.125% of the initial stock, or 10 μg/cm^2 for G98A and 5 μg/cm^2 for Ranger, no difference was detected ($P > 0.05$; data not shown).

Trichome Extract Analysis and Confirmation of Major Trichome Components. GC indicated two compounds were in common among *M. sativa* trichome extracts from the resistant G98A, less resistant G98C, and susceptible Ranger (Figure 3,

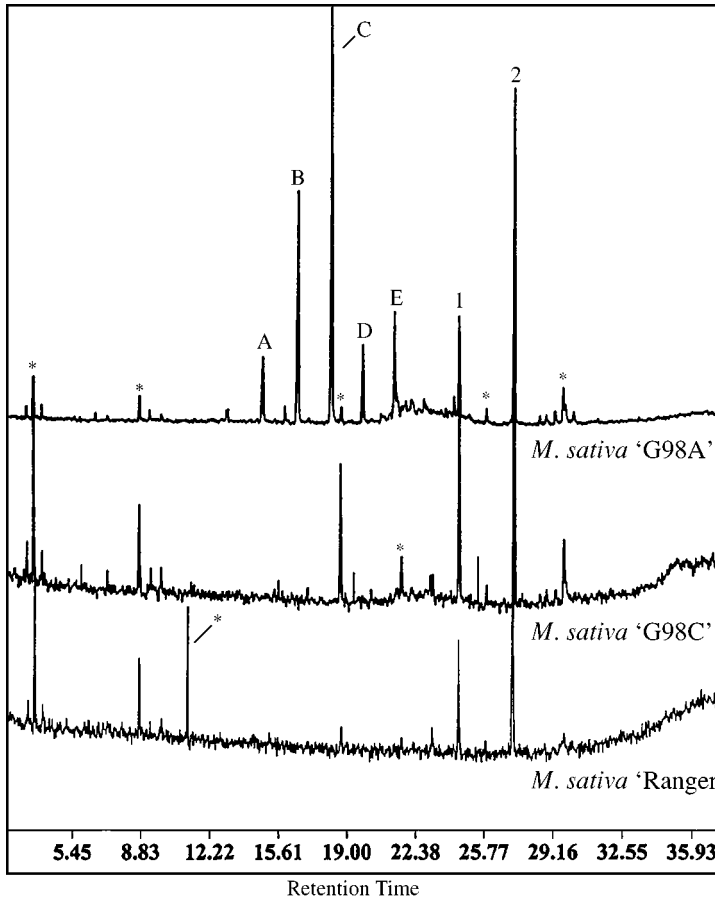

FIG. 3. GC–MS analysis of trichome extracts from highly resistant glandular-haired genotype G98A, less resistant glandular-haired genotype G98C, and susceptible nonglandular cultivar Ranger. Lettered peaks indicate fatty acid amides. Numbered peaks indicate saturated linear hydrocarbons common in all three extracts and * indicates instrument noise or phthalate contamination. The mass spectral detector was operated in electron impact mode.

peaks 1–2). Electron impact mass spectrometry (EI-MS) indicated the compounds were linear hydrocarbons and/or related alcohols.

Five additional major compounds were unique to G98A glandular trichome extracts (Figure 3, peaks A–E). All compounds exhibited odd molecular ions under EI-MS conditions, along with high mass fragments that were mainly even (Table 1). This fragmentation pattern indicated the presence of an odd number of nitrogen atoms. Furthermore, a base peak of m/z 129 is characteristic

TABLE 1. ELECTRON IMPACT MASS SPECTRA OF MAJOR COMPONENTS UNIQUE TO THE TRICHOMES OF THE GLANDULAR-HAIRED *M. sativa* G98A

Peak[a]	Retention time	*m*/*z* (relative intensity)
Fatty Acid Amide-A	14.891	297(8), 282(6), 268(3), 254(8), 242(9), 212(2), 198(5), 184(5), 170(6), 156(6), 130(8), 129(100), 128(21), 127(3), 115(86), 100(11), 98(8), 88(9), 87(10), 86(19), 85(14), 73(57), 72(18), 71(22), 70(13), 69(17), 60(24), 58(17), 57(42), 56(10), 55(36), 44(19), 43(48), 42(9), 41(30)
Fatty Acid Amide-B	16.603	311(6), 296(5), 282(2), 268(5), 256(7), 255(2), 254(2), 242(6), 240(2), 226(3), 212(2), 198(3), 184(4), 170(4), 156(4), 130(8), 129(100), 128(12), 116(5), 115(60), 114(13), 100(6), 98(5), 97(5), 88(9), 87(6), 86(15), 85(9), 142(23), 73(46), 72(13), 71(20), 70(8), 69(12), 60(16), 58(9), 57(28), 56(7), 55(24), 44(14), 43(38), 42(7), 41(21)
Fatty Acid Amide-C	18.252	325(4), 310(4), 282(3), 268(3), 256(8), 240(2), 226(2), 198(3), 184(3), 170(2), 156(3), 142(22), 130(9), 129(100), 128(2), 115(6), 114(12), 100(4), 98(3), 97(3), 88(8), 87(6), 86(13), 85(5), 73(41), 72(8), 71(14), 70(5), 69(8), 58(6), 57(15), 56(4), 55(16), 44(11), 43(27), 42(3), 41(14)
Fatty Acid Amide-D	19.814	339(6), 324(4), 310(2), 296(3), 284(4), 270(6), 267(2), 254(2), 226(2), 198(4), 184.30(3), 170(2), 156.20(4), 149(2), 142(26), 130(8), 129(100), 128(11), 127(3), 115(43), 114(12), 100(8), 98(5), 97(6), 88.15(9), 87(8), 86(16), 85(11), 73(44), 72(11), 71(20), 70(7), 69(15), 58(11), 57(31), 56(5), 55(23), 44(14), 43(36), 42(7), 41(11)
Fatty Acid Amide-E	21.357	353(4), 338(4), 324(1), 310(3), 297(2), 296(3), 293(4), 284(7), 268(2), 267(3), 254(2), 240(1), 226(1), 212(1), 198(3), 184(3), 170(2), 167(4), 156(3), 149(26), 142(21), 130(10), 129(100), 128(3), 127(5), 115(5), 114(12), 100(4), 98(5), 97(6), 88(9), 86(14), 85(12), 73(37), 71(24), 72(8), 70(10), 69(13), 58(7), 57(23), 56(6), 55(21), 44(13), 43(34), 42(5), 41(18)

[a] Peaks correspond to Figure 3.

of each of the metabolites, and is considered to correspond to the odd electron cationic species $C_5H_{11}NHCOHCH_2$. A regular difference of 14 amu between the high mass fragments was observed (Table 1), indicating the fragmentation of a long, unbranched chain. Therefore, EI-MS analyses indicated the compounds are C_5 amides with a straight fatty acid chain of C_{14} through C_{18}.

Fast atom bombardment mass spectrometry (FAB-MS) provided accurate mass measurements of the unique G98A trichome components, which allowed

TABLE 2. ACCURATE MASS RECORDINGS OF THE MAJOR COMPONENTS UNIQUE TO GLANDULAR TRICHOMES OF *M. sativa* G98A

Peak[a]	Formula[b]	Calculated mass	Observed mass	Error (ppm/mmu)
Fatty Acid Amide-A	$C_{19}H_{40}NO$	298.3110	298.3089	−7.0/−2.1
Fatty Acid Amide-B	$C_{20}H_{42}NO$	312.3266	312.3274	+2.5/+0.8
Fatty Acid Amide-C	$C_{21}H_{44}NO$	326.3423	326.3425	+0.3/+0.1
Fatty Acid Amide-D	$C_{22}H_{46}NO$	340.3579	340.3577	−0.6/−0.2
Fatty Acid Amide-E	$C_{23}H_{48}NO$	354.3736	354.3737	+0.2/+0.1

[a] Peaks correspond to Figure 3.
[b] Identifications are based on high-resolution fast atom bombardment mass spectrometry. Listed formulas are the only ones which have mass values within 10 ppm of the experimental values under the following limits: up to 50 C's, 120 H's, 5 O's, and 3 N's.

for the calculation of molecular formulas (Table 2). Results from FAB-MS corresponded with structural deductions made from EI-MS in that the unique G98A components are a homologous series of fatty acid amides $C_nH_{2n+1}NO$ (where $n = 19–23$).

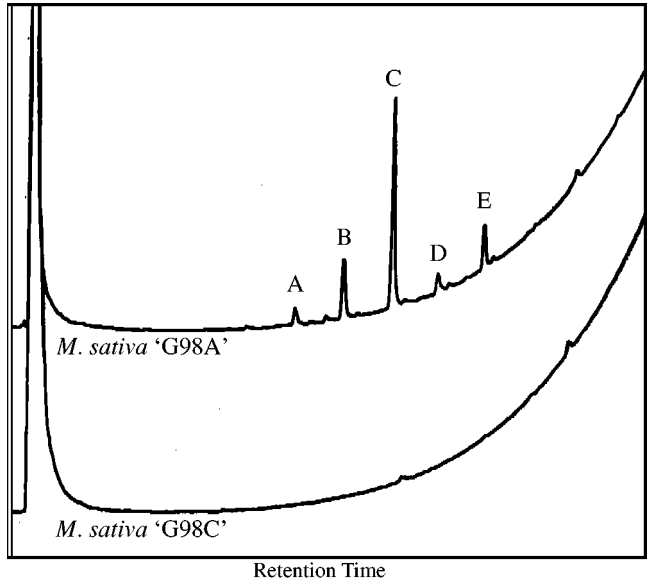

FIG. 4. GC-FID analysis of the major components of erect glandular trichomes from highly resistant glandular-haired genotype G98A and less resistant glandular-haired genotype G98C. Individual erect glandular trichomes were extracted with a finely drawn pipette using methylene chloride. See Methods and Materials for details. Lettered peaks indicate fatty acid amides unique to G98A.

Fatty acid amides were confirmed as the major components in the erect glandular trichomes from *M. sativa* G98A by the extraction of 500 individual gland heads. GC analysis of the individual trichome extracts revealed five major peaks (Figure 4). Ratios of the compounds were similar to the five lipophilic amides identified from crude *M. sativa* G98A trichome extracts (Figures 3 and 4). Furthermore, retention times of the five compounds isolated via capillary extraction corresponded to the fatty acid amides found in crude extracts from resistant *M. sativa* G98A. The lipophilic amides also were not detected in gland head extracts from less resistant G98C (Figure 4), which matches the analyses of isolated trichome extracts (Figure 3).

DISCUSSION

Wink (1999) stated it is advantageous for plants to store biologically active metabolites within glandular trichomes. For instance, herbivorous insects frequently come in direct contact with plant tissues, and this is particularly true for the bodies of small insects encountering glandular trichomes and/or an exudate (Berenbaum, 1986). With respect to *Medicago* spp. and genotypes, previous studies have suggested that the glandular trichomes secrete compounds responsible for potato leafhopper resistance (Shade et al., 1979; Hogg and McCaslin, 1994; Elden and McCaslin, 1997; Ranger and Hower, 2001a, 2002; Shockley et al., 2002; Shockley and Backus, 2002). However, preference studies presented herein provide the first direct evidence that compounds localized in the glandular trichomes of *M. sativa* G98A affect settling behavior of the potato leafhopper.

An increase in concentration of G98A extracts resulted in decreased acceptance by the leafhopper, documenting a dose response. Crude *M. sativa* G98A extracts were not highly deterrent at 10 and 25 μg/cm^2 (data not shown), which may be attributed to active compounds in the crude extracts being present only in low concentrations. The number of leafhoppers settling on the *M. sativa* Ranger extracts also increased with a decrease in concentration. However, in each of the dose-response bioassays, solvent control was not significantly preferred over crude Ranger trichome extracts. Concentration influences whether a compound has a deterrent, phagostimulant, or neutral property (Lewis and van Emden, 1986).

Chemical analysis of glandular trichome extracts from elite *M. sativa* genotypes has not been reported in the literature. However, Triebe (1981) used GC–MS to examine erect glandular trichome exudate from annual *M. scutellata* (L.) and perennial *M. sativa* subsp. *praefalcata* (Sinskaya). Nonvolatile aldehydes, alkanes, and esters were associated with the trichome exudate. Analysis of *M. sativa* G98A glandular trichome extracts also revealed the presence of large, nonvolatile compounds. In particular, a homologous series of fatty acid amides $C_nH_{2n+1}NO$ ($n = 19$–23) were isolated from the glandular trichomes (Figures 3 and 4, Tables 1 and 2). Capillary extraction of 500 individual trichomes confirmed that these

lipophilic amides were the major constituents of the extracts (Figure 4). Volatile compounds were not detected in *M. sativa* trichome extracts from resistant G98A, less resistant G98C, or susceptible Ranger.

The presence of nonvolatile compounds in trichome extracts from G98A suggests the potato leafhopper needs to touch the exudate in order for resistance to occur. Behavioral deterrence may, thus, be due to lipophilic trichome compounds, such as fatty acid amides (Tables 1 and 2), interacting with the leafhopper's contact chemoreceptors. Behavioral studies revealed that third instar potato leafhoppers cleaned their tarsi with an excretory droplet more frequently following contact with *M. sativa* glandular trichomes (Ranger and Hower, 2002). In addition, lipophilic compounds have been shown to possess deterrent properties towards insect herbivores. Goffreda et al. (1989) isolated and purified 2,3,4-tri esters of glucose with C_4–C_{12} fatty acid chain lengths from glandular trichome exudate of *Lycopersicon pennellii* Corr. (D'Arcy). In two-choice bioassays, the purified glucose esters deterred settling of the potato aphid, *Macrosiphum euphorbiae* Thomas, at concentrations ranging from 25 to 200 $\mu g/cm^2$.

Smith (1989) stated that plant antixenosis could be due to phytochemicals that repel or deter insect herbivores from feeding or oviposition, along with the presence of physical barriers such as trichomes. Preference studies conducted with intact plant material have demonstrated that adult potato leafhoppers avoid GH genotypes (Hogg and McCaslin, 1994; Ranger and Hower, 2002; Shockley and Backus, 2002). Studies presented here document that the potato leafhopper also avoids trichome extracts from GH *M. sativa* G98A. Therefore, resistance of GH *M. sativa* genotypes at least partly involves an antixenotic mechanism, which is the result of chemical and morphological barriers presented by the trichomes. Avoidance of GH *M. sativa* genotypes by the potato leafhopper may explain the decreased survivorship when leafhoppers are confined with no-choice to GH genotypes (Hogg and McCaslin, 1994; Elden and McCaslin, 1997; Ranger and Hower, 2002; Shockley and Backus, 2002). Under no-choice conditions, mortality may arise due to behavioral deterrence, rather than toxicity (Renwick, 1983). For instance, Shockley et al. (2002) found decreased feeding levels by adult potato leafhoppers caged with GH genotypes in a no-choice situation, compared with a susceptible control.

It is possible that glandular trichome exudate from *M. sativa* genotypes detrimentally affects the potato leafhopper's physiology, in addition to its host-selection behavior. After adhering to the leafhopper's cuticle, the lipophilic compounds may be absorbed and subsequently cause toxicity or a sublethal effect. Lipophilic amides isolated from *Piper nigrum* L. produced various degrees of contact toxicity to several insect herbivores (de Paula et al., 2000). Examination of the toxicity of *M. sativa* glandular trichome extracts, and the role of the lipophilic amides in conferring resistance, is in progress.

In general, the experiments presented here provide a better understanding of the types of compounds localized in *M. sativa* glandular trichomes and the

importance of behavioral deterrence in resistance of GH *M. sativa* to the potato leafhopper. This information may lead to advancements in future breeding and genetic engineering programs. Bernays and Chapman (1987) have cautioned that plant compounds functioning solely as behavioral deterrents will ultimately fail to maintain protection against insect herbivory. This should be considered during the selection process for improved genotypes.

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IDENTIFICATION OF VOLATILE SYNOMONES, INDUCED BY *Nezara viridula* FEEDING AND OVIPOSITION ON BEAN spp., THAT ATTRACT THE EGG PARASITOID *Trissolcus basalis*

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Abstract—Bean plants (*Vicia faba* L. and *Phaseolus vulgaris* L.) damaged by feeding activity of *Nezara viridula* (L.) (Heteroptera: Pentatomidae), and onto which an egg mass had been laid, produced volatiles that attracted the egg parasitoid *Trissolcus basalis* (Wollaston) (Hymenoptera: Scelionidae). Extracts of volatiles of broad bean and French bean plants induced by adults of *N. viridula* as a result of their feeding activity, oviposition activity, and feeding and oviposition activity combined were analyzed by gas chromatography–mass spectrometry (GC–MS), and tested in Y-tube olfactometer bioassays as attractants for *T. basalis* females. In extracts from undamaged leguminous plants, green-leaf volatiles were absent or scarcely detected, and monoterpenes and sesquiterpenes were present at trace levels. No significant differences were detected in the profiles of volatiles of undamaged plants, and undamaged plants on which bugs were allowed only to lay eggs. In contrast, feeding and oviposition by adults of *N. viridula* induced in both leguminous plants a significant increase in terpenoids such as linalool, (*E*)-β-caryophyllene, (*E*, *E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, and (3*E*)-4,8-dimethyl-1,3,7-nonatriene, which was induced only in French bean plants. Quantitative comparisons revealed increased levels of (*E*)-β-caryophyllene in extracts from feeding-damaged plants with *N. viridula* egg masses compared to feeding-damaged plants without egg masses. In Y-tube olfactometer bioassays, *T. basalis* females were attracted by extracts of both leguminous plants only when *N. viridula* adults were allowed to feed and oviposit upon them. Fractionation of extracts of volatiles from broad bean plants with *N. viridula* feeding damage and egg masses yielded two fractions,

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but only the fraction containing (*E*)-β-caryophyllene was attractive to the egg parasitoid. These findings indicate that *N. viridula* feeding and oviposition induce leguminous plants to produce blends of volatiles that are characterized by increased amounts of (*E*)-β-caryophyllene, and these blends attract female *T. basalis*. The role of (*E*)-β-caryophyllene as a potential synomone for *T. basalis* is discussed.

Key Words—Legume, Insecta, Heteroptera, Pentatomidae, Scelionidae, egg parasitoid, oviposition-induced synomone, terpenoid, (*E*)-β-caryophyllene.

INTRODUCTION

Volatile cues emitted by plants as a result of herbivore feeding and oviposition can play a key role in providing information to carnivorous insects (predators and parasitoids) to assist them in locating prey and hosts, respectively. As a consequence, such volatiles indirectly reduce further damage to the plants themselves (Vinson, 1985; Turlings et al., 1995; Turlings and Benrey, 1998; Dicke, 2000; Dicke and van Loon, 2000; Gatehouse, 2002 and references therein). Many parasitoids are known to discriminate between volatile chemicals produced by undamaged plants versus plants infested with a particular herbivorous species (Steinberg et al., 1993; Tumlinson et al., 1993b). The induced odors act as cues, leading female parasitoids to the habitat of their hosts (Turlings et al., 1990; Tumlinson et al., 1993b). These volatiles, named herbivore-induced synomones, are regarded as an indirect phytochemical defense that assists plants in protecting themselves from herbivore damage (Turlings et al., 1995; Dicke, 1999; Gatehouse, 2002). From a biochemical point of view, the induction of volatiles results in an alteration of the plant's constitutively emitted blend (quantitative change), and/or in a production of novel compounds (qualitative change) (Paré and Tumlinson, 1997; De Moraes et al., 1998; Boland et al., 1999). Induced changes in profiles of volatiles can occur locally or systemically, and may lead to intraindividual variation in addition to constitutive variations (Turlings and Tumlinson, 1992; Karban and Baldwin, 1997; Dicke, 1999).

In the last few years, these types of compounds have been demonstrated in more than 15 different tritrophic systems, suggesting that herbivore-induced synomones are a common phenomenon, with possible applications in biological control programs (Cortesero et al., 2000; Degenhardt et al., 2003). To date, research has focused primarily on defoliating insects that damage plants by chewing, such as caterpillars (e.g., Potting et al., 1995; De Moraes et al., 1998), or by feeding on cell contents, such as mites (Takabayashi and Dicke, 1996). Furthermore, for hymenopteran parasitoids, the role played by herbivore-induced synomones in host location has been documented mainly for parasitoids of adult and larval stages, that is, for those host instars whose feeding activities can induce both qualitative and quantitative changes in the plant's volatile profile (Dicke and van Loon, 2000). There is less information about tritrophic systems comprised of herbivores

with phloem-feeding or stylet-sheath-feeding habits, such as Heteroptera and Homoptera, and their parasitoids (Bernasconi et al., 1998; Du et al., 1998; Turlings et al., 1998; Guerrieri et al., 1999; Rodriguez-Saona et al., 2002). These feeding habits generally result in limited mechanical tissue damage to plants, and as a result, plant responses to feeding by these herbivores may be somewhat different from the case of defoliators. Furthermore, changes in plant volatiles have been documented to occur as a result of egg deposition by herbivores, and these volatiles also can act as host-induced synomones for egg parasitoids (Meiners and Hilker, 2000; Hilker and Meiners, 2002; Hilker et al., 2002a; Colazza et al., 2004). It is advantageous for egg parasitoids to be able to detect volatiles induced in plants soon after herbivore eggs are laid (Vinson, 1998), and the induced plants also increase their fitness by recruiting parasitoids to attack the herbivore eggs before significant damage to the plant has occurred, that is, before the herbivore eggs have hatched (Hilker et al., 2002a).

To date, oviposition-induced synomones have been reported only from three different tritrophic systems. Two of these, the perennial plants, *Ulmus minor* Mill. and *Pinus sylvestris* L., respond to oviposition of their herbivores, *Xanthogaleruca luteola* Muller (Coleoptera: Chrysomelidae) and *Diprion pini* (L.) (Hymenoptera: Diprionidae), respectively, by emitting volatiles that attract specialist egg parasitoids of these herbivores, *Oomyzus gallerucae* (Fonscolombe) (Hymenoptera: Eulophidae) and *Chrysonotomyia ruforum* (Krausse) (Hymenoptera: Eulophidae) (for review see Hilker and Meiners, 2002). The third tritrophic system consists of the annual plants *Vicia faba* L. and *Phaseolus vulgaris* L., which, under the combined feeding and oviposition activity by a piercing/sucking herbivore, *Nezara viridula* (L.) (Heteroptera: Pentatomidae), emit volatiles that attract the egg parasitoid *Trissolcus basalis* (Wollaston) (Hymenoptera: Scelionidae) (Colazza et al., 2004). *N. viridula* is a polyphagous herbivore with a worldwide distribution that feeds on both perennial and annual plants. It is a pest of an array of economically important crops, including grains, legumes, and other vegetables, fruits, cotton, and nut crops (Todd, 1989). *Trissolcus basalis* attacks several pentatomid bug species (Colazza and Bin, 1995), and it is also distributed worldwide as a result of extensive introductions as a biological control agent for *N. viridula* (Jones, 1988). Previous investigations have shown that *T. basalis* females are attracted to volatiles of leguminous plants induced by *N. viridula* feeding and oviposition activity (Colazza et al., 2004). Furthermore, the induced volatiles were released both locally and systemically, and plants were no longer attractive to *T. basalis* once the egg masses hatched (Colazza et al., 2004).

Here, we present the results of coupled gas chromatography–mass spectrometry (GC–MS) analyses of the volatiles released into the headspace of two bean *spp*., broad bean (*V. faba*) and French bean (*P. vulgaris*.). Volatiles were collected from plants that had been damaged by feeding, oviposition, or both feeding and oviposition of *N. viridula* adults. Extracts were assessed in Y-tube bioassays for

their attractiveness to foraging female *T. basalis*. Attractive extracts of broad bean plants damaged by both feeding and oviposition were then fractionated to identify the attractive components.

METHODS AND MATERIALS

Insect Cultures. *Nezara viridula* and *T. basalis* cultures were started from insects collected from weeds and field crops around Riverside, CA. The parasitoid and bug colonies were reared in two separate controlled environment chambers at $25 \pm 2^{\circ}$C, $60 \pm 5\%$ RH, and 16L:8D. The *N. viridula* colony was held in a wooden cage ($76 \times 46 \times 46$ cm), and fed with raw peanuts, sunflower seeds, organically grown green beans, and alfalfa bouquets in water. Eggs were collected daily and held on filter paper until hatch. Adult *T. basalis* were kept in plastic vials (7 cm $\times$ 2.5 cm ID) and fed with a honey-water solution. Single *N. viridula* egg masses were exposed to 3–4 female wasps daily, then the parasitized egg masses were stored in a large glass jar at 75% RH located in the same controlled environment room until adult emergence. In preparation for bioassays, 2–3-d old females, naive with respect to the cues released by leguminous plants and *N. viridula* adults, were individually isolated in a small vial with a drop of honey-water solution and acclimatized in the bioassay room for $\sim$24 hr before use.

Voucher specimens of the insects used in the experiments are deposited in the Entomology Museum (University of California, Riverside) (*N. viridula* voucher numbers: UCRCENT87109 to 87114; *T. basalis* voucher numbers: UCRCENT87115 to 87124).

Growing and Treatment of Plants. Broad bean plants (*V. faba* L., cv. Broad Windsor, Bay Farm Services, Bay City, MI) and French bean plants (*P. vulgaris*, cv. Blue Lake 274, bush, Ferry Morse Seed Co.) were used in all experiments. Plants were grown from seeds sown in plastic pots (8×10 cm) with fertilized commercial potting soil (Supersoil Potting Soil) at a density of 2 plant/pot (broad bean) or 1 plant/pot (French bean) in a greenhouse.

Plants 2-3-wk old with at least two (French bean, $\sim$65 cm^2 total leaf surface) or six (broad bean, $\sim$180 cm^2 leaf surface) fully expanded leaves were transferred into a climate-controlled chamber ($25 \pm 2^{\circ}$C, $45 \pm 5\%$ RH, and 16L:8D) for the treatments and for the collection of headspace volatiles. Plants were either left as controls or subjected to the following treatments: feeding-damaged plants (French bean = 13 replicates; broad bean = 12 replicates); feeding-damaged plants with egg mass (French bean = 12 replicates; broad bean = 12 replicates); undamaged plants with egg mass (broad bean = 7 replicates). Plants damaged by *N. viridula* feeding were obtained by placing 5–6 adult bugs ($\sim$6–10-d old; mixed sexes) on each plant, whereas to obtain feeding-damaged plants with egg masses, 5–6 gravid, mated females were placed on plants and allowed to oviposit one or two egg masses

per plant altogether. Bugs were allowed to feed on the plants for 24 hr, and the plants were then transferred to volatiles collection chambers (see below). To obtain undamaged plants with egg masses, the mouthparts were excised from a group of mated females 24 hr prior to placing them on plants. Only plants onto which an egg mass had been laid were used for the collection of volatiles. Control plants from the same cohorts as the treated plants (French bean = 13 replicates; broad bean = 12 replicates) were of the same age and size as treated plants, and were transferred into the climate-controlled room at the same time as the treatments.

Plant Volatile Collection System. Two-piece cylindrical glass chambers (40 cm × 13 cm ID; internal volume = ∼5.3 l) with an O-ring-sealed middle joint were used to collect headspace volatiles from the plants. After each collection, chambers were washed, rinsed with acetone, and baked overnight at ∼200°C. Single-potted plants were placed in each aeration chamber, with the pot and soil wrapped with aluminum foil to reduce contamination from soil odors. Air purified by passage through an activated charcoal filter was passed through the chambers at 4 l/min. Plant volatiles were trapped on a collector made from a glass tube (7.5 cm × 0.5 cm ID) containing ∼0.10 g of activated charcoal (50–200 mesh, precleaned by thermal desorption at 200°C under N_2; Fisher Scientific, USA) packed between two glass wool plugs. Volatiles were sampled for 20 hr. Parasitoid females respond to the cues emitted by treated plants throughout the photophase (Colazza and Pompanon, 1994; Colazza et al., 2004). The volatiles collection period was chosen to include most of this activity period. To minimize variation due to changes in diel plant volatile emission patterns, all headspace collections were started at 14:00 hr and stopped at 10:00 hr the next day. Shorter volatiles collection periods produced too little sample for analysis or bioassays. To monitor the volatile emissions of broad bean plants over a period of several days after being damaged by *N. viridula* feeding and oviposition, 20-hr collections were made from five plants of each treatment over three sequential days, i.e., 0–20, 24–44, and 48–68 hr after the plants were placed in the aeration chamber.

Traps were extracted with 200 μl CH_2Cl_2 containing two internal standards (30 ng each of toluene and methyl phenylacetate in 30 μl CH_2Cl_2). Extracts were stored at –20°C in glass vials with Teflon cap-liners until used for analysis.

Analysis of Extracts of Volatiles. One microliter of each extract was analyzed on an Hewlett-Packard (H-P) 5890 gas chromatograph (GC) equipped with a thick-film DB-1 column (30 m × 0.32 mm ID, 3 μm film; J&W Scientific, Folsom, CA) and with a flame ionization detector (FID). Injector and detector temperatures were 250 and 275°C, respectively. Injections were made in splitless mode at 250°C with a constant column flow of 35 cm/sec helium. The oven was programmed from 35°C/min, 5°C/min to 250°C for 20 min. Data were collected with H-P 3396 integrating chart recorder, and the detected volatiles were quantified based on comparison of their peak areas with those of the internal standards. To confirm identifications, selected samples were analyzed with an H-P 6890 GC

coupled to an H-P 5973 mass selective detector, and their mass spectra and retention times were matched with those of authentic standards. Standards of hexanal, (*E*)-2-hexenal, (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate, linalool, myrcene, tridecane, and (*E*)-β-caryophyllene were obtained from Aldrich, Sigma, or Fluka Chemical Co. A mixed standard of octane, (*E*,*E*)-α-farnesene, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene was a gift from Dr James Tumlinson, Pennsylvania State University, College Park, PA.

Preparative GC. Three selected samples of plant volatiles of feeding-damaged broad bean plants with egg masses were combined, and 100 μl of the mixture were separated into two fractions by five sequential injections of 20 μl of combined extract. Fractionation was carried out with an H-P 5890 GC equipped with a glass column packed with 10% SP-1000 on 80/100 Supelcoport (2 m $\times$ 2 mm ID; Supelco, Bellefonte, PA) and with a flame ionization detector (FID). Injector and detector temperatures were 200°C. The oven was programmed from 50°C/min, 10°C/min to 200°C for 20 min. The column effluent was split between a 0.1 mm ID fused silica capillary to the FID, and a 0.53 mm ID fused silica capillary to the collection port, for a split ratio of $\sim$1:28. Compounds were trapped in 15 cm $\times$ 2 mm ID silanized capillary tubes cooled in the middle with a chunk of dry ice. Trapped volatiles were eluted with about 20 μl of CH_2Cl_2. The total collection time was 40 min, with the first fraction constituting the first 13 min, and the second from $\sim$13 min until the end of the run. Fractions from five separate injections were combined, and the crude extracts and fractions were reanalyzed on capillary GC as described above to determine their contents and concentrations. Mixed extracts and fractions were stored at –20°C in glass vials with Teflon cap-liners until used for bioassays.

Y-Tube Olfactometer and Bioassay Procedure. Wasps' responses to extracts of plant volatiles and to the two preparative GC fractions of extracts of feeding-damaged broad bean plants with egg masses were investigated with a dual choice Y-tube olfactometer. The arena consisted of a thick rectangular sheet of polycarbonate (190 $\times$ 200 $\times$ 10 mm thick) with a Y-shape cut out of the center (central arm 90 mm long, two lateral arms 80 mm long at 130° angle; width of each arm 15 mm) sandwiched between two glass plates (Colazza et al., 1997). A 10 mm diam hole was drilled through the block into the end of each arm to allow air lines to be connected, and to allow test organisms to be introduced into the bottom end of the central arm. A stream of medical air humidified by bubbling through a gas washing bottle was drawn through each arm of the olfactometer at 0.15 l/min per arm. Four halogen lamps (Chroma 50, 20W) illuminated the olfactometer. In all bioassays, 11 mm red rubber sleeve stoppers loaded with 5 μl of extract solutions (corresponding to the volatiles collected in 2 hr) were used as test stimuli. Stimuli were randomly assigned to each arm at the beginning of the bioassays, replaced after testing with two wasps, and their positions were reversed after every sixth wasp to minimize any positional bias. To bioassay plant volatiles, three

extracts were randomly selected from each plant species (French and broad beans) subjected to each of the treatment or control regimes, and 36 wasps were tested with each pair of test stimuli. To test the fractions obtained from feeding-damaged broad bean plants with egg masses, 12 wasps were tested for each combination. Tests were conducted from 09:00 hr to 16:00 hr. Each time the test stimulus positions were reversed, the glass tubes holding the test lures were changed and the walls of the Y-shaped arena were wiped with solvent and air dried before reuse. At the end of the day, the whole system was thoroughly washed, and the glass parts were rinsed in acetone and baked overnight at 200°C. The olfactometer was cleaned with a laboratory detergent, rinsed with hot tap water followed by distilled water, and air dried at room temperature. Female wasps were tested one at a time, introducing individuals into the olfactometer at the entrance of the stem and observing their behavior for 5 min. Behaviors were recorded on video (monitor Sony Trinitron; B/W CCD camera Sanyo VCB-3512T, Sony TV zoom lens 12.5–75 mm/F1.8), and a video frame grabber (Studio PCTV—Pinnacle Systems, Mountain View, CA) was used to digitize the analog video signals. Xbug, a custom-designed video tracking and motion analysis software, was used to process data (sample rate = ~6 images/sec) (Colazza et al., 1999a,b). To record and calculate the total time that a wasp spent in each olfactometer arm, we defined a hypothetical line in each arm, 5 mm distal to the central Y-junction. The temperature in the bioassay room was ~26°C.

Statistical Analysis. Differences among the mean amounts of individual plant volatiles found in extracts from plants subjected to the different treatments were analyzed by a completely randomized one-way ANOVA. When differences were detected, means were separated by a Student—Newman–Keuls pairwise multiple comparison procedure. Values for residence times of parasitoids in each arm of the Y-tube olfactometer were compared with *t*-tests for paired samples. All data were analyzed with the program SPSS for Windows 11.0 (SPSS Inc., 2001).

RESULTS

Volatile Compounds from Untreated and Treated Bean Plants. Undamaged French bean and broad bean plants emitted nearly the same compounds, with the exception that three unidentified compounds were obtained only from broad bean plants, and (*E*)-2-hexenal, *n*-octane, and (3*E*)-4,8-dimethyl-1,3,7-nonatriene were obtained only from French bean plants (Figure 1, Table 1). Because of the differences in the total leaf surface areas between French bean and broad bean plants (total leaf surface = ~65 and ~180 cm^2, respectively), comparisons of the total amount of volatiles released by each species were not made.

There were significant differences in the total amounts of volatile emissions from undamaged and treated French bean plants. Volatile emissions increased

TABLE 1. MEAN AMOUNTS (±SD) OF VOLATILES COLLECTED FROM BROAD BEAN (*V. faba*) AND FRENCH BEAN (*P. vulgaris*) PLANTS THAT WERE UNDAMAGED (Und), OR ON WHICH *N. viridula* ADULTS HAD FED (FedNegg), LAID AN EGG MASS (Nfedegg), OR FED AND OVIPOSITED COMBINED (Fedegg)[a]

		Compounds (ng)[b]													
		Aldehydes		Unidentified					Ester				Terpenoids		
Plant/ Odor source	*N*	Hexanal	(*E*)-2-Hexenal	1	2	3	Hydrocarbon Octane	Alcohol (*Z*)-3-Hexenol	(*Z*)-3-Hexenyl-acetate	Myrcene	Linaool	DMNT[c]	(*E*)-β-Caryophyllene	(*E*,*E*)-α-Farnesene	TMTT[c]
V. fava															
Und	12	8.03 ± 5.02a	—	13.24 ± 9.64a	11.99 ± 7.12a	75.36 ± 44.02a	—	231.17 ± 131.99a	36.66 ± 29.95a	0.35 ± 0.73a	2.81 ± 1.24a	—	1.83 ± 1.36a	0.94 ± 2.75a	2.65 ± 2.98a
FedNegg	12	9.54 ± 3.47a	—	12.91 ± 6.25a	7.14 ± 6.04ab	72.15 ± 34.55a	—	231.01 ± 99.83a	41.86 ± 30.39a	0.49 ± 0.92a	4.20 ± 1.42b	—	5.91 ± 3.99a	1.99 ± 3.60a	17.55 ± 9.12b
Fedegg	12	10.22 ± 6.61a	—	11.65 ± 14.67a	5.38 ± 6.32b	47.33 ± 42.01a	—	164.75 ± 148.55a	24.21 ± 15.16a	1.28 ± 1.05a	6.02 ± 3.23b	—	13.35 ± 11.40b	1.19 ± 1.25a	17.49 ± 10.90b
Nfedegg	7	11.50 ± 6.91a	—	15.36 ± 10.69a	3.87 ± 2.14b	52.08 ± 26.17a	—	185.02 ± 98.65a	33.15 ± 40.88a	0.33 ± 0.57a	3.16 ± 1.23a	—	1.23 ± 1.19a	0.36 ± 0.64a	4.81 ± 5.84a
P. vulgaris															
Und	13	1.15 ± 0.69a	1.33 ± 0.89a	—	—	—	0.00 ± 0.00a	1.05 ± 0.50a	0.43 ± 0.87a	1.44 ± 0.65a	2.70 ± 1.23a	0.56 ± 0.48a	0.02 ± 0.10a	0.36 ± 0.37a	0.37 ± 0.44a
FedNegg	13	1.99 ± 1.04a	2.18 ± 1.50a	—	—	—	0.21 ± 0.22b	2.71 ± 2.14a	11.23 ± 12.14ab	1.79 ± 1.20a	5.90 ± 2.88b	14.47 ± 16.03b	0.34 ± 0.49a	1.02 ± 0.33a	14.93 ± 18.54b
Fedegg	12	2.70 ± 1.23a	1.32 ± 0.80a	—	—	—	0.19 ± 0.24b	5.21 ± 5.68b	24.62 ± 26.07b	1.19 ± 1.27a	7.98 ± 3.28b	17.55 ± 14.04b	3.78 ± 6.84b	3.22 ± 5.52a	12.71 ± 11.93b

[a] Collections lasted 20 hr starting at 4:00 P.M.

[b] N = Number of replicates. Average amount were calculated based on the peak area of an internal standard (toluene). Average numbers in columns for each plant followed by the same letter were not significantly different ($P > 0.05$, ANOVA).

[c] *DMNT* = (3*E*)-4,8-Dimethyl-1,3,7-nonatriene; *TMTT* = (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene.

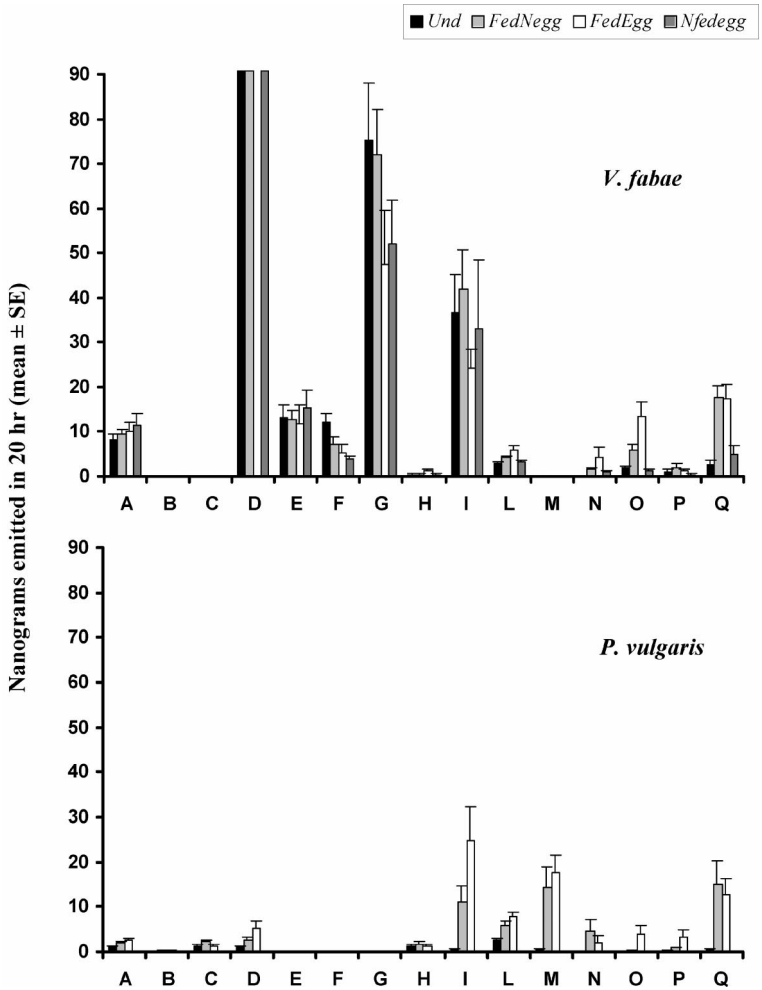

FIG. 1. Quantification of the major volatiles collected from undamaged (Und) broad bean plants (*V. fabae*) and French bean plants (*P. vulgaris*) and from plants on which *N. viridula* adults had fed (FedNegg), oviposited (Nfedegg), or fed and oviposited combined (Fedegg). Compounds are listed based on their GC retention times. Each bar represents the mean ± SE. The amounts of (*Z*)-3-hexenol emitted by broad bean plants (Und = 231.2 ± 38.1; FedNegg = 231.0 ± 28.8; FedEgg = 164.8 ± 42.9; Nfedegg = 185.0 ± 37.3) are off scale in order to show minor compounds accurately. A = hexanal, B = octane, C = (*E*)-2-hexenal, D = (*Z*)-3-hexenol, E = unknown 1, F = unknown 2, G = unknown 3, H = myrcene, I = (*Z*)-3-hexenyl acetate, L = linalool, M = (3*E*)-4,8-dimethyl-1,3,7-nonatriene, N = tridecane, O = (*E*)-β-caryophyllene, P = (*E*, *E*)-α-farnesene, Q = (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene.

seven- to ninefold in feeding-damaged plants and feeding-damaged plants with egg masses as compared to untreated plants ($N = 38$, $F_{2,35} = 13.72$, $P < 0.001$, ANOVA). Insect activities also affected the quality of the induced odor blend due to the production of octane, a compound that was not detected from undamaged plants (Table 1). Tridecane was also detected in the volatiles blend emitted by French bean plants damaged by feeding (Figure 1). However, tridecane is a major component of *N. viridula* metathoracic gland secretions and its presence in the extracts of treated plants may be attributable to its retention and slow reemission from the plant's epicuticular waxes. In addition, there were quantitative changes in the headspace among the different plant treatments. Specifically, feeding-damaged plants and feeding-damaged plants with egg masses released more (*Z*)-3-hexenol ($N = 38$, $F_{2,35} = 4.61$, $P = 0.017$, ANOVA), (*Z*)-3-hexenyl acetate ($N = 38$, $F_{2,35} = 6.90$, $P = 0.003$, ANOVA), linalool ($N = 38$, $F_{2,35} = 12.96$, $P < 0.001$, ANOVA), (3*E*)-4,8-dimethyl-1,3,7-nonatriene ($N = 38$, $F_{2,35} = 6.93$, $P = 0.003$, ANOVA), (*E*)-β-caryophyllene ($N = 38$, $F_{2,35} = 3.60$, $P = 0.03$, ANOVA), and (*E*, *E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene ($N = 38$, $F_{2,35} = 4.88$, $P = 0.01$, ANOVA) (Table 1). Furthermore, the combined activity of feeding and oviposition by *N. viridula* on French bean induced larger amounts of (*Z*)-3-hexenol and (*E*)-β-caryophyllene compared to plants damaged only by feeding (Table 1).

In contrast to French bean, in extracts from broad bean plants, all compounds detected in the headspace of undamaged plants were found in the treated plants (Table 1) with the exception of tridecane, whose presence may be artifactual as described above (Figure 1). No differences were observed in the total amount of volatiles collected comparing undamaged plants versus treated plants ($N = 43$, $F_{3,39} = 0.65$, $P = 0.58$, ANOVA). In particular, the odor profiles of undamaged broad bean plants and plants without feeding damage but carrying *N. viridula* egg masses were similar (Table 1). However, qualitative changes were observed between undamaged broad bean plants and broad bean plants damaged by feeding activity of *N. viridula* and feeding-damaged plants with egg masses. Plants damaged by *N. viridula* feeding activity and feeding-damaged plants with egg masses produced more of unknown compound 2 ($N = 43$, $F_{3,39} = 3.54$, $P = 0.023$, ANOVA), and lesser amounts of terpenoids such as linalool ($N = 43$, $F_{3,39} = 5.57$, $P = 0.003$, ANOVA), (*E*)-β-caryophyllene ($N = 43$, $F_{3,39} = 8.08$, $P < 0.001$, ANOVA), and (*E*, *E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene ($N = 43$, $F_{3,39} = 10.92$, $P < 0.001$, ANOVA) (Table 1). Interestingly, in feeding-damaged plants with egg masses, only the terpenoid (*E*)-β-caryophyllene was significantly higher in quantity compared to plants with only feeding damage (Table 1).

The induced emission of terpenoids in broad beans occurred rapidly (Figure 2). In both feeding-damaged plants and feeding-damaged plants with egg masses, the aggregate amounts of terpenoids had already significantly increased in the first collection period (0–20 hr after treatment). In the second (24–44 hr after treatments) and third collections (48–68 hr after treatments), the emission

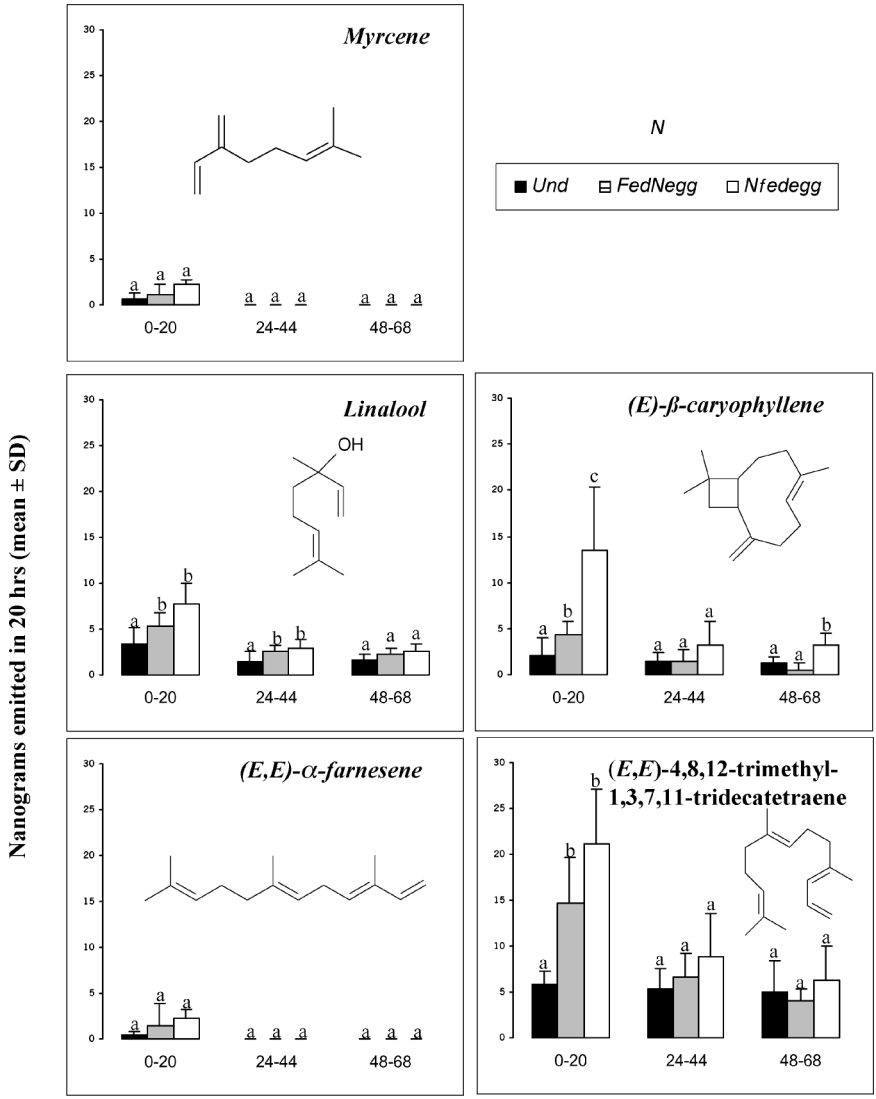

FIG. 2. Mean amount (±SD) of total terpenoids released during three consecutive volatile collection periods (0–20, 24–44, 48–68) of 20 hr each from undamaged (Und) broad bean plants (*V. fabae*), or from plants on which *N. viridula* adults had fed (FedNegg) or laid an egg mass (Nfedegg). Experiments were replicated five times. Different letters within each group of three bars represent significant differences in mean amounts of compounds collected (Student–Newman–Keuls tests after ANOVA).

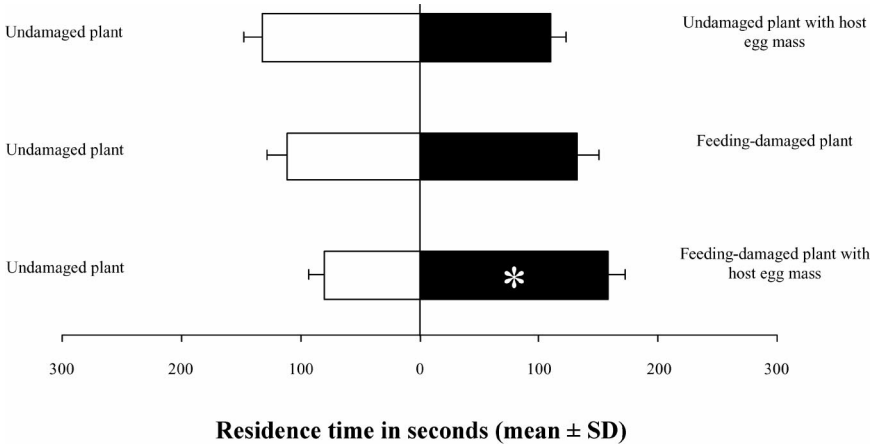

FIG. 3. Responses of *T. basalis* females in a Y-tube olfactometer to extracts of *V. faba* plants. Bars indicate mean values (±SD) of the time spent in each arm of the olfactometer by 36 females over an observation period of 300 sec. Asterisks indicate significant differences between treatments at $P < 0.01$ (paired t-tests).

of terpenoids dropped, but in feeding-damaged plants with egg masses, (*E*)-β-caryophyllene was still present in higher levels during the third collection period compared to undamaged plants and feeding-damaged plants (Figure 2).

Behavioral Responses of Parasitoids in Y-Tube Olfactometer. In dual choice Y-tube olfactometer bioassays, *T. basalis* females spent more time in the arm with the volatiles from extracts of feeding-damaged broad bean plants with egg masses than in the arm with volatiles of undamaged broad bean plants ($t = 3.23$, df $= 35$, $P = 0.002$; paired t-test) (Figure 3). In contrast, female wasps did not discriminate between the extracts from feeding-damaged broad bean plants and extracts from undamaged plants with host egg masses ($t = 0.69$, df $= 35$, $P = 0.49$; $t = -0.93$, df $= 35$, $P = 0.35$ respectively; paired t-test) (Figure 3), spending similar amounts of time in each arm of the Y-tube.

Similar results were obtained when the wasps were exposed to the extracts of French bean plants (Figure 4). Female wasps showed a preference for extracts of feeding-damaged French bean plants carrying an egg mass both when they were tested versus extracts of undamaged French bean plants ($t = 3.88$, df $= 35$, $P < 0.001$; paired t-test), and when they were tested versus extracts of feeding-damaged French bean plants ($t = 4.78$, df $= 35$, $P < 0.001$; paired t-test). Extracts of French bean plants with only feeding damage were no more attractive to the wasps than extracts from undamaged control plants ($t = 0.37$, df $= 35$, $P = 0.71$; paired t-test).

Trissolcus basalis females responded positively to a combined sample of three extracts from feeding-damaged broad bean plants with egg masses ($t = 3.65$,

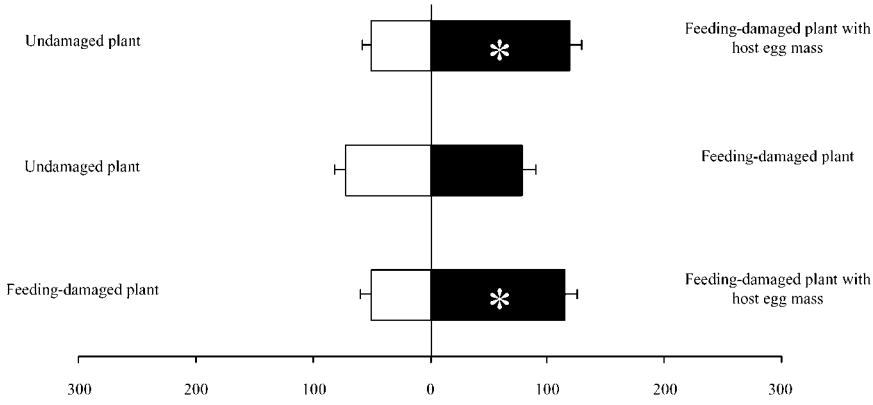

FIG. 4. Responses of *T. basalis* females in a Y-tube olfactometer to extracts of *P. vulgaris* plants. Bars indicate mean values (±SD) of the times spent in each arm by 36 females over an observation period of 300 sec. Asterisks indicate significant differences between treatments at $P < 0.01$ (paired *t*-tests).

df = 17, $P = 0.001$; paired *t*-test) (Figure 5). This combined extract was fractionated by preparative GC, and the relative amounts of volatiles present in the crude extract and in the two fractions are shown in Table 2. Bioassays performed with these fractions revealed that female wasps responded most strongly to fraction B ($t = 3.90$, df = 17, $P = 0.001$; paired *t*-test), whereas fraction A was less attractive to female wasps than extracts of undamaged broad bean plants ($t = -2.28$, df = 17, $P = 0.03$; paired *t*-test) (Figure 5).

DISCUSSION

Host selection behaviors of hymenopteran parasitoids proceed through a series of chemically mediated steps that bring female parasitoids into proximity or contact with their potential hosts (Vinson, 1998). For parasitoids attacking herbivores, potential cues to locate host community include volatiles released by plants, both directly from wounding and indirectly from induction, as a result of herbivore attack (Tumlinson et al., 1993a; Turlings et al., 1995; Dicke, 1999, 2000). Furthermore, recent work has shown that production of volatiles may be induced by oviposition as well as by feeding and, just as larval- and/or adult-feeding-induced volatiles attract parasitoids of larval or adult stages, respectively, so oviposition-induced volatiles can attract egg parasitoids (Hilker et al., 2002b; Hilker and Meiners, 2002; Colazza et al., 2004).

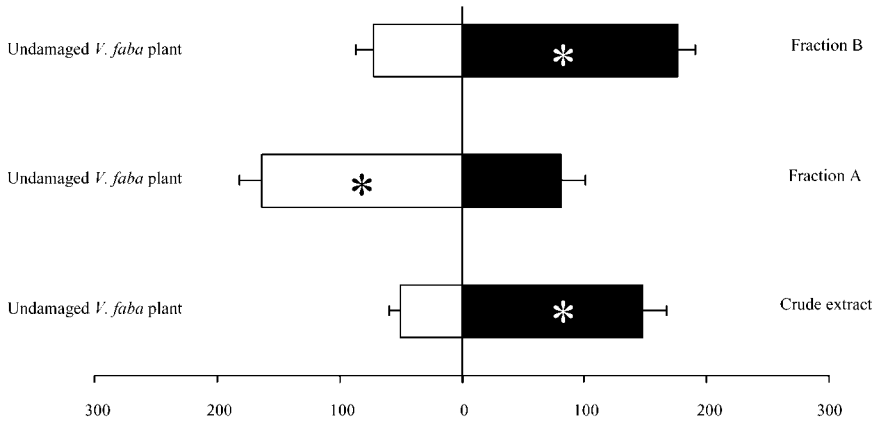

FIG. 5. Responses of *T. basalis* females in a Y-tube olfactometer to odors from a combined crude extract of plant volatiles from three feeding-damaged broad bean plants with egg masses and from two preparative GC fractions of the crude extract (A and B). Bars indicate mean values (±SD) of the times spent in each arm by 12 females over an observation period of 300 sec. Asterisks indicate significant differences between treatments at $P < 0.01$ (paired *t*-tests).

TABLE 2. RELATIVE AMOUNTS OF VOLATILES COLLECTED FROM THREE COMBINED CRUDE EXTRACTS OF FEEDING-DAMAGED BROAD BEAN PLANTS WITH EGG MASSES, AND TWO PREPARATIVE GC FRACTIONS FROM THIS COMBINED EXTRACT

Compound	Relative amounts		
	Crude extract	Fraction A	Fraction B
Hexanal	47	26	—
(*Z*)-3-Hexenol	590	611	—
Unknown 1	32	33	—
Unknown 2	11	—	—
Unknown 3	199	—	100
(*Z*)-3-Hexenyl acetate	99	103	—
Linalool	16	13	—
(*E*)-β-Caryophyllene	75	—	73
(*E*,*E*)-α-Farnesene	3	—	4
(*E*,*E*)-4,8,12-Trimethyl-1,3,7, 11-tridecatetraene	74	—	75

Data presented here confirm that *N. viridula*-infested bean plants with egg masses release volatile compounds that attract the egg parasitoid *T. basalis*. Under our experimental conditions, bean plants damaged by feeding by *N. viridula* did not produce elevated levels of green-leaf volatiles. The green-leaf volatiles constitute a blend of saturated and unsaturated six-carbon alcohols, aldehydes, and esters produced by the lipoxygenase pathway, and they are typically released by leaves that have been mechanically damaged, for example, by the feeding of defoliating herbivores (Turlings et al., 1998; Paré and Tumlinson, 1999). Headspace analyses of broad bean plants showed that the amounts of (*Z*)-3-hexenol and (*Z*)-3-hexenyl acetate in undamaged control plants and plants upon which *N. viridula* (a sucking insect rather than a chewing insect) had fed and oviposited were unchanged. Similarly, headspace analyses of control or feeding-damaged French bean plants produced low to scarcely detectable levels of green-leaf volatiles, with no differences between insect-infested and control plants. This lack of induction of green-leaf volatiles by *N. viridula* is analogous to results obtained in experiments with other sucking insects feeding on similar plants. For example, broad bean plants infested by the pea aphid, *Acyrthosiphon pisum* (Harris) (Homoptera: Aphididae), and maize plants infested by *Rhopalosiphum maidis* (Fitch) (Homoptera: Aphididae) were not induced to produce high levels of green-leaf volatiles, even at very heavy infestation levels (Du et al., 1998; Turlings et al., 1998). Maize and cotton plants infested by *Lygus hesperus* Knight (Hemiptera: Miridae) did produce significantly more (*Z*)-3-hexenal and (*Z*)-3-hexenyl acetate than insect-free plants (Rodriguez-Saona et al., 2002), but these results were obtained with very high infestation levels (30–40 adults/plant), and with the insects feeding and ovipositing on the plants during collection of volatiles. Overall, the absence of induction of green-leaf volatiles production in the bean plants used in our experiments in response to *N. viridula* infestation suggests that the minimal amount of mechanical damage caused by stink bug feeding does not activate the lipoxygenase pathway.

Comparison of the pattern of terpenes between control and treated plants revealed quantitative rather than qualitative differences. Feeding activity of *N. viridula* increased the relative amounts of the monoterpene linalool and the C_{16} homosesquiterpene (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene in both plant species, and the C_{11} homomonoterpene 4,8-dimethyl-1,3(*E*),7-nonatriene only in French bean. Similar induction of homoterpenoids was induced by *L. hesperus* in cotton and maize (Rodriguez-Saona et al., 2002). Many insect-induced terpenoids are known to require a latency period between the initial stimulus and the resulting increase in the *de novo* biosynthesis. Compounds such as (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene and (3*E*)-4,8-dimethyl-1,3,7-nonatriene are induced by herbivore damage in many different tritrophic systems (Baldwin and Preston, 1999; Paré and Tumlinson, 1999), and these compounds are generally synthesized *de novo* by the plants within hours of feeding (Paré and Tumlinson, 1997). In

particular, (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene has been hypothesized to be derived from geranyllinalool, a diterpene analog of nerolidol, and it is also a constituent of herbivore-induced volatile blends of other plant species such as corn and cotton (Gäbler et al., 1991). Furthermore, Donath and Boland (1995) demonstrated that several plant species can convert the acyclic sesquiterpene alcohol (3*S*)-(*E*)-nerolidol to (3*E*)-4,8-dimethyl-1,3,7-nonatriene. Therefore, on the basis of our results it seems likely that there are subtle differences between French bean and broad bean in the activities of the enzymes involved in the homoterpenoid biosynthetic pathways.

Both leguminous plants showed quantitative differences in their odor blends when *N. viridula* feeding activity was combined with oviposition on leaf surfaces. In particular, the plants emitted more (*E*)-β-caryophyllene in response to the deposition of egg masses. Several studies have shown increased production of this sesquiterpenoid in plants induced by mechanical damage and/or by herbivore feeding damage (Turlings et al., 1998; Agelopoulos et al., 1999; Wegener et al., 2001; Gouinguene and Turlings, 2002; Rodriguez-Saona et al., 2002). Interestingly, both herbivorous and carnivorous insects use (*E*)-β-caryophyllene as a cue in different contexts. For example, both the weevil *Pissodes notatus* (F.) (Coleoptera, Curculionidae) (Bichao et al., 2003) and the parasitoid *Campoletis sonorensis* (Cameron) (Hymenoptera: Ichneumonidae) are attracted by (*E*)-β-caryophyllene (Elzen et al., 1984). This sesquiterpene also attracted predatory green lacewings, *Chrysopa carnea* Step. (Neuroptera: Chrysopidae), in field bioassays (Flint et al., 1979). Ongoing research on the chemistry of the volatiles induced by insect oviposition on living plants is demonstrating that insect egg deposition *per se* can induce both qualitative and/or quantitative changes in host plant odors. For example, elm leaves with *X. luteola* eggs produce blends of volatiles that are qualitatively and quantitatively different than the blends resulting from *X. luteola* feeding damage (Wegener et al., 2001). Three new homoterpenoids, (*E*)-2,6-dimethyl-6,8-nonadien-4-one, (*E*)-2,6-dimethyl-2,6,8-nonatrien-4-one, and (3*R*,6*E*)-2,3-epoxy-2,6-dimethyl-6,8-nonadiene, have recently been identified from elm leaves with *X. luteola* eggs, and their role in the attraction of the egg parasitoid is under investigation (Wegener and Schulz, 2002). Furthermore, quantitative comparison of the odor blend from pine twigs induced by *D. pini* oviposition versus odors from undamaged twigs revealed that only the amounts of (*E*)-β-farnesene were significantly elevated as a result of egg-laying (Mumm et al., 2003). However, in both these cases, oviposition involves some physical damage to the host plant because eggs are laid in slits excised in the plant tissues. In contrast, *N. viridula* eggs are simply deposited in clusters directly onto the plant surface, with no mechanical damage to the plant tissues (Colazza et al., 2004).

In summary, data presented here confirm that *N. viridula*-infested bean plants with egg masses release volatile compounds that attract the egg parasitoid *T. basalis*. These findings, and those from previous investigations (Mattiacci et al., 1993; Colazza et al., 1999a,b; Colazza et al., 2004) suggest that *T. basalis* females

use odors from *N. viridula* adults and from feeding-damaged plants carrying host eggs as host-location cues. Odors from adult bugs, released in relatively large amounts, may be more important for longer-range attraction, indicating the presence of potential hosts nearby. Once in the host habitat, parasitoids may then utilize the relatively small amounts of volatiles emitted by the plant as a result of adult feeding activity and oviposition as cues for host location. The combination of these two strategies may have evolved in *T. basalis*, thus overcoming the obstacle of finding eggs of herbivores attacking several different plant species. The attraction of *T. basalis* to extracts of feeding-damaged plants with egg masses suggests that (*E*)-β-caryophyllene is a synomone, beneficial to both emitter and receiver. Thus far, the mechanisms that stimulate the wasp to be attracted to elevated levels of plant odors that are induced by insect feeding and oviposition have not been investigated. Studies to investigate whether wasps respond to a specific plant compound, such as (*E*)-β-caryophyllene that is released in elevated amounts, or whether blends with a particular mixture and ratio of components are more important, are currently under investigation.

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LABORATORY AND FIELD RESPONSES OF THE MOSQUITO, *Culex quinquefasciatus*, TO PLANT-DERIVED *Culex* spp. OVIPOSITION PHEROMONE AND THE OVIPOSITION CUE SKATOLE

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Abstract—Laboratory and field studies were conducted on the oviposition behavior of the pathogen-vectoring mosquito, *Culex quinquefasciatus*, in response to the oviposition pheromone 6-acetoxy-5-hexadecanolide, produced from a renewable plant resource, *Kochia scoparia* (Chenopodiaceae) (plant-derived pheromone, PDP), and *via* an established synthetic route (synthetic oviposition pheromone, SOP). Responses to the oviposition cue skatole (3-methylindole), presented individually and in combination with the plant-derived and synthetic oviposition pheromone, were also studied. Both laboratory and field assays showed that PDP and SOP were equally attractive. Synergistic effects were observed with one combination of PDP and skatole combinations in laboratory assays. Synergy was also observed under field conditions. SOP and skatole combinations showed additive effects in laboratory assays, but were not tested in field bioassays. Although synergism has been previously demonstrated with combinations of SOP and polluted waters, the work presented here is the first example of synergy between a specific oviposition attractant and the oviposition pheromone. Furthermore, the efficacy of mosquito pheromone produced from a cheap, renewable botanical source has been demonstrated.

Key Words—*Culex quinquefasciatus*, oviposition, pheromone, 6-acetoxy-5-hexadecanolide, *Kochia scoparia*, renewable resource, skatole, synergism.

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INTRODUCTION

Mosquitoes (Diptera, Culicidae) represent a significant threat to human health because of their ability to vector pathogens that cause diseases that afflict millions of people worldwide (WHO, 1992; Pinheiro, 1997; WHO/CTD, 1998). In addition to those major areas where mosquito-vectored diseases are currently endemic, in particular sub-Saharan Africa, problems may arise in new areas as consequences of changes in global climate patterns and increased global travel and trade (Donaldson, 2002). Thus, strategies for improved vector surveillance, as well as direct control, have been sought for the major mosquito vectors. Such strategies include the application of semiochemicals, particularly those involved in mediating oviposition site location behavior.

Culex spp. mosquitoes are responsible for the transmission of a number of pathogens, notably *Wucheria bancrofti*, the causative agent for urban bancroftian filariasis, and arboviruses including St. Louis encephalitis virus (Riesen et al., 1992), and more recently West Nile virus in urbanized areas in the United States and Europe (Jonsson and Reid, 2000; Turell et al., 2002). Several studies have reported the roles and identities of semiochemicals mediating oviposition behavior for *Culex* spp. mosquitoes. The oviposition pheromone, (5*R*,6*S*)-6-acetoxy-5-hexadecanolide, was originally identified over 20 years ago (Laurence and Pickett, 1982), and field trials in several countries in afflicted areas have since demonstrated the efficacy of synthetic pheromone in the field (e.g., Mboera et al., 2000a,b). Site-derived oviposition cues have been identified, with skatole (3-methylindole) being the most active component in the laboratory (Millar et al., 1992; Mordue (Luntz) et al., 1992; Blackwell et al., 1993) and in the field (Beehler et al., 1994). Recent field studies in Tanzania have shown that a potent signal for oviposition site selection comprises a synergistic combination of the pheromone and grass infusions or soakage pit water (Mboera et al., 1999, 2000b). For the development of optimized monitoring or control strategies, the use of individual components identified from organically enriched water would be advantageous, because the use of infusions and water samples with variable and undefined levels of oviposition cues could potentially lead to erratic responses (Mordue (Luntz) et al., 1992; Blackwell et al., 1993). Thus, the oviposition pheromone and skatole have been used together effectively in the field (Mbeora et al., 2000a). The attractive effect of these components suggests that they could be used in the development of control strategies, which include the use of either environmentally benign larvicides, such as the insect growth regulator pyriproxyfen, larvae-specific pathogens, such as the fungus *Lagenidium giganteum* Couch (Pickett and Woodcock, 1996), or trapping systems (Mboera et al., 2000b). Though many synthetic routes for production of multigram quantities of the pheromone have been published (see Olagbemiro et al., 1999, and references therein), the costs and hazardous nature of reagents have prevented large-scale pheromone production, particularly in less developed countries. It was

demonstrated recently that the pheromone could be produced rapidly, cheaply, and efficiently from a botanical source of (*Z*)-5-hexadecenoic acid, found as a minor component of the fixed seed oil of the summer cypress plant, *Kochia scoparia* (Chenopodiaceae) (Olagbemiro et al., 1999).

To confirm that pheromone production from *K. scoparia* seed oil could be considered as a viable option for cheap pheromone production, the objective of the work reported here was to assess the biological activity of plant-derived pheromone (PDP) in inducing oviposition by gravid female *Cx. quinquefasciatus* mosquitoes when compared to synthetic oviposition pheromone (SOP) prepared using an established route (Dawson et al., 1990), using a previously established laboratory oviposition bioassay (Blackwell et al., 1993). To demonstrate that plant-derived pheromone material was also active in the field, studies were conducted at urbanized oviposition sites in Bauchi State, Nigeria. Field bioassays were also conducted to compare the effect of skatole as an oviposition cue for *Cx. quinquefasciatus* in Western Africa, with that previously reported in Tanzania (Mboera et al., 2000a), and to determine its interaction with plant-derived pheromone compared to synthetic pheromone.

METHODS AND MATERIALS

Chemicals

Skatole (99%, 3-methylindole) was purchased from the Aldrich Chemical Co., Gillingham, UK. Solutions of skatole in hexane were prepared for laboratory bioassays, while a stock solution (100 mg) in 96% ethanol (100 ml) was used to prepare aqueous solutions for field studies. The synthetic oviposition pheromone (SOP) material was prepared using a previously established route (Dawson et al., 1990), and comprised a 1:1:1:1 mixture of the four stereoisomers of 6-acetoxy-5-hexadecanolide, i.e., 25% of the material consisted of the biologically active (5*R*,6*S*)-enantiomer. The plant-derived pheromone (PDP) material was produced from the seed oil of the summer cypress plant, *Kochia scoparia* (Chenopodiaeae), by a route previously reported (Olagbemiro et al., 1999). The oil contained the precursor (*Z*)-5-hexadecenoic acid in minor amounts (approximately 7%), and was used directly in pheromone synthesis without purification of the precursor. Intermediates were used at every stage without purification, and intermediate formation was confirmed by $^{1}H/^{13}C$ NMR and GC analysis. Thus, the final PDP material contained the pheromone mixed with the other components in the seed oil, and included a 1:1 ratio of the active (5*R*,6*S*)-enantiomer and the inactive (5*S*,6*R*) isomer, with the pair comprising approximately 27% (w:w) of the PDP material, i.e., approximately 13.5% of each. As SOP and PDP were minor components in their respective materials, amounts of material were used in bioassays such that 5 μg and 5 mg doses of the active (5*R*,6*S*)-enantiomer were used per laboratory

and field experiment, respectively. Solutions of PDP and SOP in hexane, with the concentration of the active (5*R*,6*S*) enantiomer at 1 mg/ml, were used for laboratory studies. For field studies, blank effervescent tablets (see Otieno et al., 1988, for formulation) were laced with pheromone material (PDP) prior to use. Tablets were spread individually on a clean piece of paper, and pheromone (5 mg active enantiomer per tablet) was added as a hexane solution (0.1 ml) to each tablet. The tablets were left to dry for a few minutes at room temperature before use. All solvents were freshly distilled prior to use.

Insect Behavior

Laboratory Bioassays. Cx. quinquefasciatus Say (Lagos strain) mosquitoes were maintained on a LD 12:12 hr photoperiod at 27 ± 2°C, 55–60% humidity. Larvae were fed daily on desiccated liver powder. Adults were fed on a sucrose solution, and females were transferred after 8 d to goose blood (Alsevers), fed through a membrane feeder (Hemotek 5W1 system, Discovery Workshops, Accrington, UK). For behavior experiments, gravid females were taken from the colony 4 d after feeding (7- to 10-d-old).

Trials were carried out in muslin-covered wooden framed cages (31 × 31 × 31 cm) with Perspex fronts and muslin sleeves. Each trial involved 20 gravid female *Cx. quinquefasciatus* and was replicated over several nights in randomized blocks. For two-choice experiments, two glass bowls containing 100 ml distilled water were placed at diagonally opposite corners of the cages. For four-choice experiments, glass bowls were placed in each of the four corners. The cages were left overnight (for 17 hr) under the same conditions as described for colony maintenance. The number of egg rafts in the bowls was recorded the following morning and converted to percentages of the total number of rafts in both bowls for each cage. Control bowls contained distilled water (100 ml) plus solvent where appropriate. Skatole and pheromone doses used were based on those shown previously to be the most effective in laboratory tests (Mordue (Luntz) et al., 1992; Blackwell et al., 1993). Skatole was applied directly to the water, and pheromone was applied to glass coverslips floated on plastic caps to simulate release from egg rafts. Thus, test bowls contained distilled water (100 ml) plus pheromone material (5 μg, PDP or SOP) and/or skatole at one of two concentrations (10^{-4} or 10^{-5} μg/l). The following experiments were conducted: (a) two-choice experiments, PDP vs. control and SOP vs. control; (b) four-choice experiments, control vs. skatole vs. PDP vs. skatole/PDP and control vs. skatole vs. SOP vs skatole/SOP. Each trial was repeated 10–20 times depending on mosquito availability.

Field Studies. All experiments were conducted in Bauchi (10°17′N, 9°49′W), Nigeria. The mean annual temperature is 38°C, and the average rainfall of 1000 mm falls mainly in a short rainy season (June–September). Twelve pit latrines were selected for the experiments, located at four sites. Each of the pit latrines consisted

of 1 m^2 slabs of concrete provided with a drop hole (12 × 22 cm) in the center, suspended over a soakage pit, and mounted on a total floor area of 4 m^2. Experiments were performed during the rainy seasons of 2001 and 2002.

In each of the trials, treatments comprised black plastic bowls containing 1000 ml of non-chlorinated water plus either skatole and/or an effervescent pheromone tablet, placed 1 m apart on the floor of a pit latrine building. Treatments alternated between the four sites for 4 nights and were rerandomized within each latrine between experiments. Experiments started at 18:00 hr local time and were stopped at 08:00 hr the following morning. In all experiments, collected egg rafts were taken to the laboratory, sorted by shape (Edwards, 1942; Gillett, 1972), and counted. The number of *Cx. quinquefasciatus* egg rafts in the bowls was converted to percentages of the total number of rafts in both bowls. Eggs from other *Culex* spp. mosquitoes were not counted in this study, which was directed exclusively to *Cx. quinquefasciatus*. The following experiments were conducted and replicated 12 times (a) response of *Cx. quinquefasciatus* to SOP, PDP, or a control. One of the bowls was treated with a control tablet (0.1 ml hexane only). The two remaining bowls were treated with SOP or PDP tablets, respectively; (b) oviposition response of *Cx. quinquefasciatus* to skatole. To determine the optimum dose of skatole at the field sites, the stock solution of skatole in ethanol was subjected to serial dilution using non-chlorinated water to make the following concentrations: 10^{-4}, 10^{-5}, 10^{-6}, and $10^{-7}\mu$g/l; (c) oviposition responses of *Cx. quinquefasciatus* to control (hexane only), PDP, skatole (10^{-5}gμg/l), and PDP + skatole (10^{-5}gμg/l).

Data Analysis

Laboratory Bioassays. In laboratory bioassays, data were analyzed by nonparametric tests because data were not normally distributed in some cases. Factorial experiments were subjected to Kruskal–Wallis analysis, followed by a Wilcoxon paired test to compare differences between treatment means. Synergism between treatments was tested using Student's *t*-test to compare the sum of effects of skatole and oviposition pheromone presented separately to the effects of skatole and pheromone presented together.

Field Studies. All field data were $\log(x + 1)$ transformed, and means of treatments were compared by Student's *t*-tests. Means of factorial experiments were subjected to ANOVA, and an F test significant at $P < 0.05$ was followed by a Least Significant Difference test to compare treatment means.

RESULTS

Laboratory Bioassays. Laboratory oviposition bioassays showed that the number of egg rafts laid by gravid *Cx. quinquefasciatus* in response to the mixture

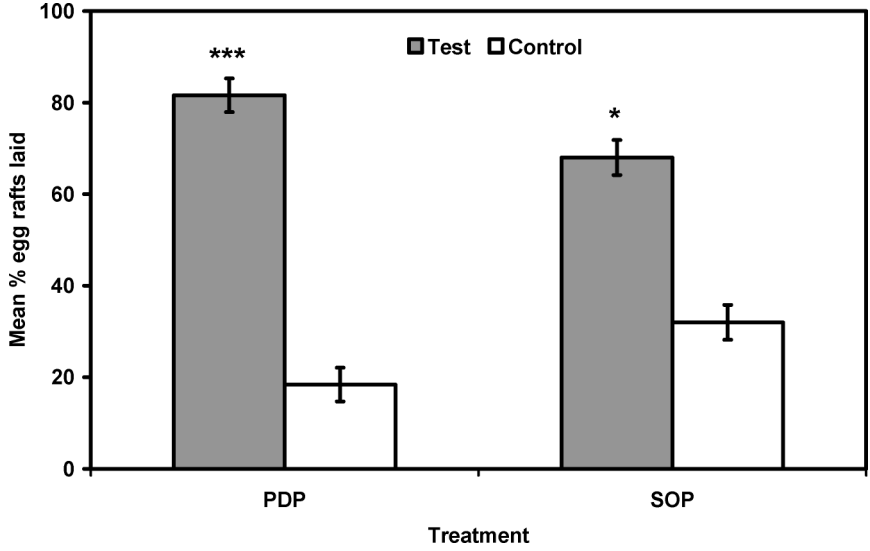

FIG. 1. Oviposition behavior of gravid females of *Culex quinquefasciatus* in the laboratory in the presence of 5 μg plant-derived (PDP) and 5 μg synthetic oviposition pheromone (SOP) ($N = 20$ and 10, respectively). Vertical bars represent standard errors. Statistical analysis was carried out using Kruskall–Wallis and Wilcoxon paired tests. $^{*}P < 0.05$ and $^{***}P < 0.001$.

of (5*R*,6*S*) and (5*S*,6*R*) stereoisomers of the oviposition pheromone 6-acetoxy-5-hexadecanolide (5 μg dose), prepared from the seed oil of *K. scoparia* (PDP) and the mixture of all four stereoisomers prepared *via* an established synthetic route (SOP), was significantly different from the control (Figure 1; PDP vs. control, mean $\pm$ SE 81.6 $\pm$ 3.7% vs. 18.4 $\pm$ 3.7%, $P < 0.001$, $N = 20$; SOP vs. control, 68 $\pm$ 3.8% vs. 32 $\pm$ 3.8%, $P < 0.05$, $N = 10$).

In multiple choice laboratory bioassays using skatole and pheromone, in the experiment using the lower skatole concentration ($10^{-5}\mu$g/l), skatole and pheromone individual treatments received more egg rafts than the control (Figure 2, control 4.3 $\pm$ 2.8%, skatole 19.6 $\pm$ 7%; $P < 0.05$, PDP 19.6 $\pm$ 6.4%; $P < 0.05$; Figure 3, control 9.7 $\pm$ 4.9%, skatole 22.2 $\pm$ 6.9%; $P < 0.05$, SOP 27.4 $\pm$ 7.3%; $P < 0.05$). Using the higher skatole concentration ($10^{-4}\mu$g/l), a similar pattern was observed (Figure 2, control 4.1 $\pm$ 2.1%, skatole 18 $\pm$ 4.1%, $P < 0.01$, PDP 14.1 $\pm$ 3.5%; $P < 0.05$; Figure 3, control 4.2 $\pm$ 2.1%, skatole 25.6 $\pm$ 3.7%; $P < 0.01$, SOP 17.2 $\pm$ 4.5%; $P < 0.05$). When skatole and pheromone treatments were combined, a difference in response was observed between PDP and SOP. For the skatole + SOP combination, additive effects were observed at both concentrations of skatole when compared to individual treatments (Figure 3, 40.7

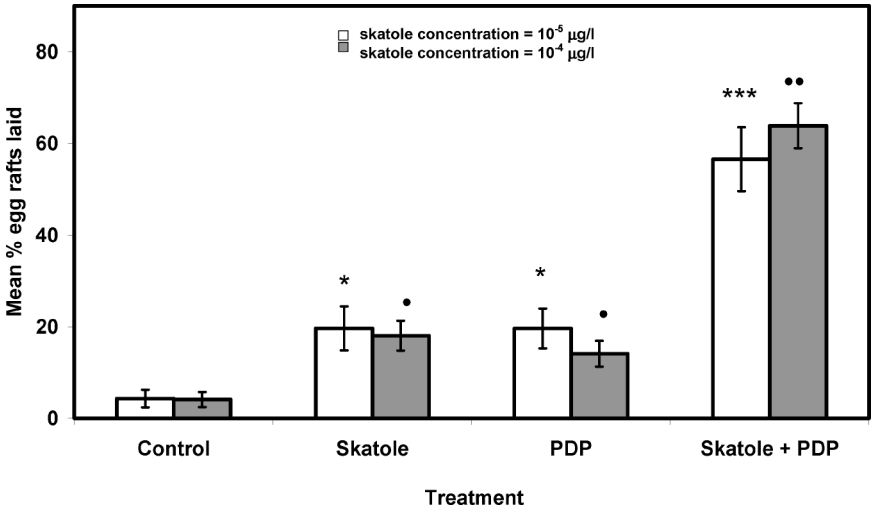

FIG. 2. Oviposition behavior of gravid females of *Culex quinquefasciatus* in the laboratory in the presence of skatole at two concentrations (10^{-4} and $10^{-5}\mu$g/l) and 5 μg plant-derived pheromone (PDP) ($N = 12$ and 16, respectively). Vertical bars represent standard errors. Statistical analysis was carried out using Kruskall–Wallis and Wilcoxon paired tests, comparing test with control data, $^{*\bullet}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

$\pm$ 6.6%; $P < 0.01$ and 53 $\pm$ 6.2%; $P < 0.01$, respectively). For skatole + PDP combinations, an additive effect was observed for the lower skatole concentration (Figure 2, 56.5 $\pm$ 10.2% vs. 39.2% for summed individual treatments; $P < 0.001$), whereas for the higher concentration of skatole, a synergistic effect was observed (Figure 2, 63.8 $\pm$ 5.9% vs. 32% for summed individual treatments, $P < 0.01$).

Field Studies. In field oviposition bioassays, no significant differences were observed in the responses of *Cx. quinquefasciatus* to PDP (46.7 $\pm$ 1.4%) and SOP (49.3 $\pm$ 1.4%) (Table 1; $P > 0.05$). Significantly fewer egg rafts were laid in control bowls containing tap water (4.8 $\pm$ 0.7%, $P < 0.05$).

In the four-choice field experiment to determine the effect of skatole concentration on oviposition, responses differed across the range of concentrations used. Concentrations of $10^{-4}\mu$g/l (31.2 $\pm$ 2.6%) and $10^{-5}\mu$g/l (37.8 $\pm$ 3.1%) were equivalent (Figure 4; $P > 0.05$) and concentrations of $10^{-6}\mu$g/l (15.2 $\pm$ 2.1%) and $10^{-7}\mu$g/l (16.1 $\pm$ 2.8%) also were not significantly different ($P > 0.05$). However, responses to the lower two concentrations were lower than the upper two concentrations ($P < 0.05$). A four-choice field experiment using skatole, PDP, skatole + PDP combination, and a control was conducted using the $10^{-5}\mu$g/l skatole concentration. The skatole treatment was different from the control (Table 2, 15.5 $\pm$ 2% vs. 5.4 $\pm$ 1.4%, $P < 0.05$) and the PDP treatment, which was no different

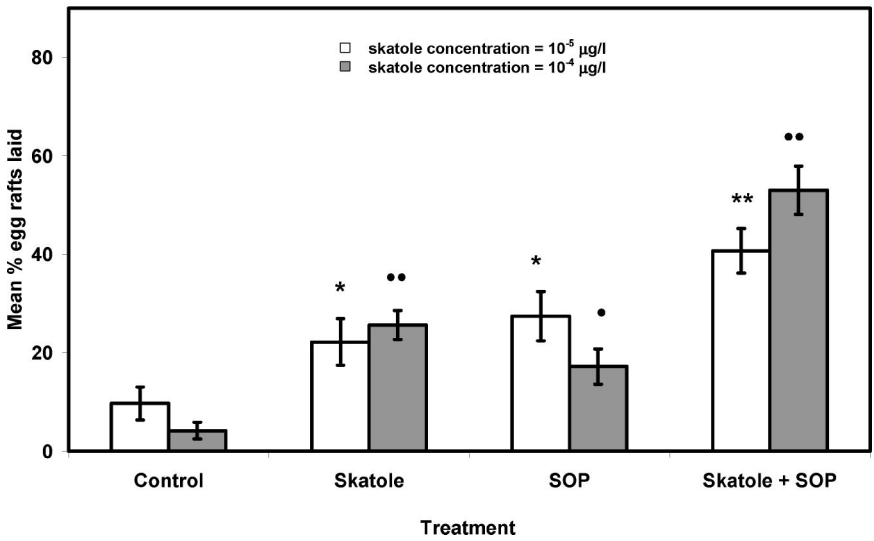

FIG. 3. Oviposition behaviour of gravid females of *Culex quinquefasciatus* in the laboratory in the presence of skatole at two concentrations (10^{-4} and $10^{-5}\mu$g/l) and 5 μg synthetic pheromone (SOP) ($N = 12$ and 16, respectively). Vertical bars represent standard errors. Statistical analysis was carried out using Kruskall–Wallis and Wilcoxon paired tests, comparing test with control data, $^{*\bullet}P < 0.05$ and $^{**\bullet\bullet}P < 0.01$.

from the control (8.3 ± 1.7%, $P > 0.05$). The skatole + PDP combination received most of the egg rafts (73.6 ± 1.8%). The substantially larger response to the skatole + PDP combination compared to the summed responses to skatole and PDP presented individually indicated synergism between the two components ($P < 0.05$).

TABLE 1. FIELD EXPERIMENT SHOWING NUMBER OF EGG RAFTS OF *Culex quinquefasciatus* DEPOSITED IN WATER TREATED WITH PLANT-DERIVED OVIPOSITION PHEROMONE (PDP) AND SYNTHETIC OVIPOSITION PHEROMONE (SOP) VERSUS TAP WATER (CONTROL)[a]

	Egg rafts	
Treatment	Total no.	Mean % ± SE
Control	75	4.8 ± 0.7 b
PDP	937	46.7 ± 1.4 c
SOP	988	49.3 ± 1.4 c

[a] $N = 12$. Means followed by a different letter are significantly different at $P < 0.05$.

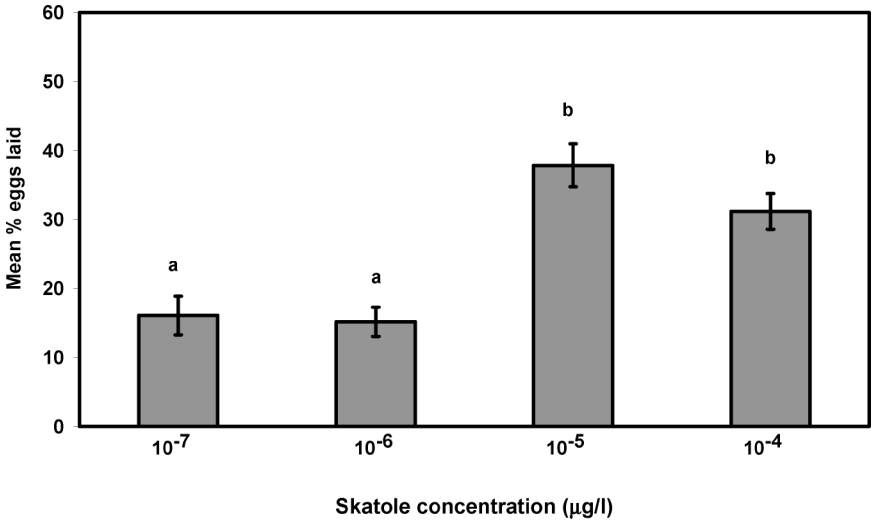

FIG. 4. The dose-response of *Culex quinquefasciatus* to skatole in four-choice bioassays in the field ($N = 12$). Vertical bars represent standard errors. Means followed by a different letter are significantly different at $P < 0.05$.

DISCUSSION

In laboratory oviposition bioassays, the pheromone prepared from a botanical precursor (PDP) was as effective in stimulating egg-laying by gravid *Cx. quinquefasciatus* mosquitoes as synthetic oviposition pheromone (SOP) prepared from fine chemicals, confirming the viability of pheromone production using the botanical route. Production of the pheromone from a precursor from renewable plant

TABLE 2. FIELD EXPERIMENT SHOWING NUMBER OF EGG RAFTS OF *Culex quinquefasciatus* DEPOSITED IN WATER TREATED WITH SKATOLE ($10^{-5}\mu$g/l), PLANT-DERIVED OVIPOSITION PHEROMONE (PDP), AND SKATOLE + PDP, VERSUS TAP WATER (CONTROL)[a]

	Egg rafts	
Treatment	Total no.	Mean % ± SE
Control	95	5.4 ± 1.4 b
Skatole	373	15.5 ± 2 c
PDP	153	8.3 ± 1.7 b
Skatole + PDP	1681	73.6 ± 1.8 d

[a]$N = 12$. Means followed by a different letter are significantly different at $P < 0.05$.

resource represents an important step forward in the development of sustainable, cheap, and efficient pheromone production.

Laboratory bioassays involving skatole and pheromone (PDP and SOP) treatments used concentrations of skatole (10^{-4} and $10^{-5}\mu$g/l) shown previously to be the most effective in increasing oviposition (Blackwell et al., 1993). An additive effect was observed for skatole + SOP combinations, in agreement with previous studies (Blackwell et al., 1993). Similar additive effects in laboratory bioassays have been observed with the pheromone and polluted water (Mordue (Luntz) et al., 1992; Blackwell et al., 1993), and the pheromone with a synthetic mixture of oviposition attractants (Millar et al., 1994). However, in this study, additive effects were obtained with PDP + skatole at one concentration ($10^{-5}\mu$g/l), whereas PDP + skatole at a higher concentration ($10^{-4}\mu$g/l) appeared to act synergistically.

Field bioassays using PDP and SOP showed no difference in the two materials, similar to the laboratory bioassays. Field assays using skatole were then conducted using a range of concentrations, to determine the optimum concentration for later use with PDP. The results indicate that skatole is used by West African populations of *Cx. quinquefasciatus* as an oviposition cue, similar to populations in Eastern Africa (Mboera et al., 2000a) and the United States (Millar et al., 1992). The dose-response study showed that the highest proportion of egg rafts of *Cx. quinquefasciatus* were laid in skatole solutions of 10^{-4} and $10^{-5}\mu$g/l, similar to the most active concentrations (10^{-5} and $10^{-6}\mu$g/l) observed in Tanzania (Mboera et al., 2000a). In contrast to field studies in Tanzania (Mboera et al., 2000a), where additive effects were observed between skatole and SOP, skatole and PDP appeared to act synergistically. Although synergy between either grass infusions or soakage pit water and SOP has been observed in the field (Mboera et al., 1999, 2000b), the work presented here is the first example of synergy between a specific mosquito oviposition attractant and the oviposition pheromone in the laboratory and in the field. Because PDP was synthesized from an unpurified precursor obtained from the seed oil of *K. scoparia* (Olagbemiro et al., 1999), it contains numerous additional components some of which may be attractive to *Cx. quinquefasciatus*. Work is underway to chemically characterize these possible additional components. It is also possible that the difference in activity of skatole and pheromone combinations in the field for West and East African populations of *Cx. quinquefasciatus* may be due to variation in the populations between the two areas (Tanzania and Nigeria).

Previous studies have shown that gravid *Cx. quinquefasciatus* are able to distinguish between sites with and without the oviposition pheromone at distances of up to 10 m in the field (Otieno et al., 1988). In pit latrines used in the field studies here, treatments were separated by a distance of only 1 m. The data in this study, which showed a much higher preference for the skatole + PDP combination compared to individual treatments, confirm that the blend of site-derived and pheromonal oviposition cues stimulate optimum oviposition activity by gravid

Cx. quinquefasciatus. Further studies to assess the distance over which skatole + PDP combinations may act will determine the potential for the use of traps at nonbreeding sites.

Oviposition attractants/stimulants have shown promise in increasing the sensitivity of gravid *Culex* mosquito traps for monitoring and control of populations in the United States (Reiter, 1983, 1986; Millar et al., 1994) and in Tanzania (Mboera et al., 1999, 2000b), respectively. The use of specific attractants such as skatole, rather than grass infusions or soakage pit water (cf. Mboera et al., 2000b), is desirable in order to standardize attractants. Accurate and reliable information regarding the breeding habits and distribution of *Cx. quinquefasciatus* mosquitoes, and *Culex* spp. mosquitoes in general, is an essential requisite for improved management strategies for these important disease vectors.

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VOLATILES ASSOCIATED WITH PREFERRED AND NONPREFERRED HOSTS OF THE NANTUCKET PINE TIP MOTH, *Rhyacionia frustrana*

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Abstract—Ovipositing female Nantucket pine tip moth, *Rhyacionia frustrana*, prefer loblolly pine, *Pinus taeda* L., to slash pine, *Pinus elliottii* Engelm. except during the first spring following planting of seedlings. Host discrimination by *R. frustrana* increases as seedlings develop, suggesting that changes in the chemical composition of seedlings may mediate the moth's host preferences. Volatile compounds from slash and loblolly pine seedlings were collected using solid-phase microextraction (SPME) during the first year following planting. Four collection periods coincided with adult emergence and oviposition during each of four annual generations of *R. frustrana* in the Georgia Coastal Plain. Infestation of slash pine peaked during the second tip moth generation and was similar to the loblolly pine infestation level. By the fourth tip moth generation, slash pine infestation levels had declined and diverged considerably from those of loblolly pine. Significant differences in relative quantities of β-pinene, α-phellandrene, limonene, β-phellandrene, bornyl acetate, β-caryophyllene, and an unidentified sesquiterpene occurred between slash and loblolly pine during the fourth generation. However, no strong correlation was observed between any individual compound and host damage that could readily explain the temporal changes in *R. frustrana* host preference. Gas chromatographic–electroantennographic detection (GC–EAD) analyses of standards identified 19 different seedling-associated compounds that elicited antennal responses from *R. frustrana* females, indicating that a blend of terpenoids may mediate host discrimination.

Key Words—Tortricidae, *Pinus taeda*, *Pinus elliottii*, terpenes, host selection, solid-phase microextraction, electroantennogram.

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INTRODUCTION

The Nantucket pine tip moth, *Rhyacionia frustrana* (Comstock) (Lepidoptera: Tortricidae), is an important pest of intensively managed loblolly pine (*Pinus taeda* L.) plantations throughout the southeastern United States. Female tip moths oviposit on needles, buds, and shoots. Larvae mine needles initially, and then bore into the bud or shoot, severing the vascular tissue and killing the apical meristem (Yates et al., 1981; Berisford, 1988; Asaro et al., 2003). Reduced growth and development of poor form may result (Cade and Hedden, 1987; Berisford et al., 1989; Nowak and Berisford, 2000; Asaro et al., 2003). *Rhyacionia frustrana* is multivoltine, with two to five generations per year in different parts of its range (Berisford, 1988; Fettig et al., 2000; Asaro et al., 2003).

Slash pine, *Pinus elliottii* Engelm., is generally resistant to *R. frustrana* attack (Yates, 1962). Hood et al. (1985) reported that *R. frustrana* oviposits almost exclusively on loblolly pine even when growing adjacent to slash pine. However, anecdotal observations suggest that slash pine seedlings are susceptible to tip moth oviposition and successful attack during the first growing season following planting with infestation rates decreasing to typical, low levels by the end of the first or second year (Yates, 1966; Hood et al., 1985; Berisford, 1988), although this has never been confirmed experimentally.

Monoterpenes are important host finding and oviposition cues for some Lepidoptera (Städler, 1974; Hanula et al., 1985; Leather, 1987; Åhman et al., 1988; Shu et al., 1997). Ross et al. (1995), using 5- to 29-month-old loblolly and slash pine seedlings, attempted to determine whether the oviposition preference of Nantucket pine tip moth was based on monoterpene emissions or cuticular lipids. They found a significantly greater amount of β-pinene and lower amounts of myrcene in slash pine compared to loblolly, whereas relative proportions among cuticular lipids varied between these two species. However, these differences have not yet been linked directly to oviposition preference.

Our primary objective was to identify olfactory cues for *R. frustrana* that could mediate this pest's ability to discriminate between slash and loblolly pine seedlings. We documented differences between the profiles of volatiles of these two host species during the first growing season following planting, and we attempted to correlate these differences with *R. frustrana* damage levels and, by association, oviposition preference during the same interval. We used headspace solid-phase microextraction (SPME) to obtain a more complete analysis of the total volatile profile of slash and loblolly pine than analyses reported previously (Ross et al., 1995). Evidence suggests that SPME is a more sensitive technique for detecting trace compounds than traditional methods for headspace sampling (Flechtmann et al., 1999; Thomsen, 1999). In addition, we used electroantennography to evaluate *R. frustrana*'s olfactory sensitivity to compounds identified in the profiles

of volatiles of the seedlings and to thereby distinguish possible semiochemicals utilized during host selection.

METHODS AND MATERIALS

Study Site. The study was conducted on two plots in Effingham County, Georgia, approximately 2 km west of Rincon, in a portion of the Georgia Coastal Plain where *R. frustrana* has four generations per year (Fettig et al., 2000). On January 5, 2000, 400 bareroot seedlings were hand planted 20 rows wide by 20 trees long with alternating rows of slash and loblolly pine at 1.8 × 3.6 m spacing on each plot. At one plot, seedlings were planted on bedded soil (soil that is mounded up into low ridges or "beds," a common forestry practice on flat, poorly drained sites). The bedded plot was row-treated with Velpar® /Oust® (Hexazinone/Sulfometuron methyl) in the spring and received a broadcast treatment of Arsenal® (Imazapyr) during summer to control competing vegetation. The unbedded plot was mowed prior to planting, and the herbicide Accord® (Glyphosate) was applied during summer in a 0.5 m circle around each tree.

Collection of Volatiles. Tree odors were collected on four dates to coincide with the adult emergence/oviposition period of each generation of *R. frustrana*: March 2, May 15, July 6, and August 25, 2000. Collections were executed during a 3-hr interval at dusk (March 2, 5–8 P.M.; May 15, 6:45–9:45 P.M.; July 6 and August 25, 7–10 P.M.) to coincide with the mating flight and oviposition of *R. frustrana* (Webb and Berisford, 1978; Berisford, 1988). Monoterpene emissions from southern pines are high during this time interval and are relatively constant over a broad temperature range (Tingey et al., 1980). Volatiles were obtained from 12 seedlings of slash and loblolly pine from each plot using SPME fibers coated with 50 μm of cross-linked divinylbenzene (DVB), carboxen, and polydimethylsiloxane (PDMS) (Supelco, Bellefonte, PA). Prior to first use, each fiber was conditioned at 270°C for 4 hr. Fibers were thermally conditioned again for 10 min at 270°C prior to each subsequent use, and gas chromatography analysis confirmed that this adequately eliminated contaminants. For sampling, each fiber was extruded and attached to a top-whorl shoot using a clothespin. A 0.95 l (17.8 × 20.3 cm) plastic (LDPE) freezer bag was placed over the shoot and fiber and partially sealed at the base with binder clips to enclose a headspace. After 3 hr, fibers were retracted into the syringe needle, sealed at the tip with a Teflon plug, placed in screw-top culture tubes, and stored on ice for transport back to the laboratory. Fibers were subsequently stored at −80°C for up to 2 wk.

Damage Estimates. At the end of each *R. frustrana* generation, top-whorl damage estimates were obtained from 20 trees of each species at each plot, including those trees sampled for volatiles. Percent damage per tree was calculated by counting the total number of shoots and damaged shoots in the top whorl, which is well correlated with whole tree damage (Fettig and Berisford, 1999; Asaro et al.,

2003). Damaged shoots were identified by the presence of a pitch mass on or near the terminal bud accompanied by dry, brown needles and buds. Because of an overlap of *R. frustrana* with the northern extreme of the range of the subtropical pine tip moth, *R. subtropica* Miller, 25 pupae were collected on October 23, 2000, from each pine species to verify that *R. frustrana* was causing the observed damage. Pupae were identified according to Yates (1967).

Chemical Analysis. Analyses were performed by desorbing each fiber in the injector of an Hewlett-Packard (H-P) GCD G1800A coupled gas chromatograph–mass spectrometer (GC–MS) equipped with an SPME inlet liner (Supelco, Bellafonte, PA) and an HP-INNOWax column (60 m $\times$ 0.25 mm i.d.; 0.33 μm film thickness) (Hewlett-Packard Corp., Palo Alto, CA). Prior to injection, each fiber sample was exposed to the equilibrated headspace of $>$98% heptyl acetate (C7Ac) within a 100 ml bottle at room temperature for 5 sec to provide a semiquantitative internal standard. Specifically, the bottle cap was removed and the opening sealed with aluminum foil. After waiting 5 min for the headspace to equilibrate with any introduced air, an SPME fiber was attached to its specialized holder (Supelco), and the needle was placed through the foil without exposing the fiber. The needle was removed and immediately inserted into the GC, and the fiber was desorbed. The repeatability of this method was confirmed (mean variation 11.6% for 10 injections).

The carrier gas was helium at a flow rate of 0.9 ml/min with a 0.7 min splitless injection time. The GC inlet temperature was 220°C, and the temperature program was 40°C for 2 min, then 16°C/min to 130°C, then 6°C/min to 210°C, then 30°C/min to 240°C for 4 min. A subsample of three fibers per sampling date were reinjected immediately after a sample run to confirm that all compounds were completely desorbed onto the column. All compound identifications were based on mass spectral and retention time matches with known standards. For statistical comparisons, headspace compounds were quantified as C7Ac equivalents (i.e., the quotient of the raw integration areas of analyte peaks within the total ion chromatograms divided by the integrated area of the internal standard C7Ac).

To estimate the absolute proportions among different compounds in the headspace samples, quantities expressed as C7Ac equivalents were corrected using response factors calculated by exposing fibers to known quantities of commercially obtained standards. Compounds for which no standard was available had response factors assigned based on their structural similarity to compounds with known response factors. For compounds whose response factors were unknown but were present in very low amounts in our field samples ($<$0.5% of the total volatile profile), no response factors were applied to raw peak areas.

Cold Storage Test. Tests were performed to determine whether cold storage of SPME fibers for up to 2 wk led to any sample loss. Ten fibers were exposed

to an evaporated pentane solution (5 μl) containing 10 ng/μl each of α-pinene, β-pinene, myrcene, α-phellandrene, terpinolene, heptyl acetate, and terpinen-4-ol within a sealed 40 ml vial for 10 min. Five of these fibers were immediately injected into the GC–MS for analysis, while other five were stored at -80°C for 2 wk and subsequently analyzed.

Electrophysiology. Gas chromatographic–electroantennographic detection (GC–EAD) analyses were performed on 14 male and 14 female *R. frustrana* that had emerged up to 5 d previously from loblolly pine shoots clipped in Oconee Co., Georgia on February 10, 2003. Prior to analyses, moths were housed in foam-plugged plastic vials with pieces of moistened paper towel at 8°C and a 14:10 (L:D) hr light regime. Electrical contact was made by inserting a glass/pipette Ag/AgCl reference electrode into a moth's excised head and inserting the distal segments of one intact antenna into a second, glass/pipette Ag/AgCl recording electrode. Both pipettes were filled with Beadle–Ephrussi saline containing 0.5% polyvinylpyrrolidone (Bjostad, 1998) and 0.01% Triton X-100 (Union Carbide Midland, MI), a wetting agent which improved saline contact with the antennal tip. The antennal preparation was positioned at the opening of a stainless steel tube (8 mm diam.) that delivered a continuous stream (400 ml/min) of charcoal filtered, humidified air.

GC–EAD analyses were carried out with an H-P GC 5890 instrument fitted with a 60 m HP-INNOWax column. The temperature program was 40°C for 1 min, then 6°C/min to 230°C. for 10 min; the injector temperature was 200°C. Effluent from the column was split 1:1 and mixed with makeup gas in a union cross (Gerstel, Berlin, Germany). Deactivated, fused silica tubing (0.32 mm diam.) delivered half of the column effluent to a flame ionization detector and the other half through a heated transfer line (240°C.; Syntech, Hilversum, The Netherlands) that exited into the stimulus delivery tube (65 mm upwind from the antennal preparation). Samples consisted of a synthetic mixture of 22 compounds (Table 1) identified in seedling foliage headspace and diluted to $\sim$45 ng/compound/μl of hexane ($\sim$90 ng/compound/μl for chiral compounds available as racemic mixtures). Samples (1 μl) were injected splitless into the GC.

Signals from the recording electrode were amplified by a high impedance guarded input AC/DC probe (Syntech) and then filtered and further amplified by an AutoSpike IDAC-2/3 signal connection interface (Syntech). Acquisition and analysis of antennal responses were performed with PeakSimple chromatography analysis software (Version 2.74) interfaced with a PeakSimple Chromatography Data System (SRI Instruments, Torrance, CA). For each run, the EAD trace was inverted, reprocessed with a moving average filter (1 sec wide), and assigned a baseline. Heights were calculated for all EAD peaks that occurred within a 23-min window that enclosed the retention times of the 22 test compounds (320–380 peaks per trial). A compound eluting from the GC was considered to have

TABLE 1. VOLATILE COMPOUNDS DESORBED FROM SPME FIBER SAMPLES FROM LOBLOLLY AND SLASH PINE

Volatile compound	Tree species
1. α-Pinene	Loblolly and slash
2. Camphene	Loblolly and slash
3. β-Pinene	Loblolly and slash
4. Sabinene[a]	Unbedded loblolly only
5. Myrcene	Loblolly and slash
6. α-Phellandrene	Loblolly and slash
7. α-Terpinene[a]	Loblolly and slash
8. Limonene	Loblolly and slash
9. β-Phellandrene	Loblolly and slash
10. γ-Terpinene[a]	Loblolly and slash
11. *p*-Cymene[a]	Slash only
12. Terpinolene	Loblolly and slash
13. Linalool	Loblolly and slash
14. Camphor[a]	Loblolly only
15. Bornyl acetate	Loblolly and slash
16. Terpinen-4-ol[a]	Loblolly and slash
17. β-Caryophyllene	Loblolly and slash
18. Myrtenal[a]	Slash only
19. *trans*-Verbenol[a]	Loblolly and slash
20. 4-Allylanisole[a]	Slash only
21. α-Terpineol[a]	Loblolly and slash
22. Borneol[a]	Loblolly and slash
23. α-Humulene	Loblolly and slash
24. Verbenone[a]	Loblolly and slash
25. Unknown sesquiterpene	Loblolly and slash

[a] Compounds found in trace amounts (<0.5% of total volatile profile).

produced a significant EAD response (i.e., one distinct from random noise), when the coinciding EAD spike fell in the 90% percentile for height more often than three times out of 14 runs ($P \leq 0.044$, table of cumulative binomial probabilities; Sokal and Rohlf, 1995).

Statistical Analyses. Damage estimates among tree species and plots were compared within generations using ANOVA followed by Tukey's test for means separation or Kruskal–Wallis ANOVA followed by Dunn's test if normality or equal variance assumptions were violated (SigmaStat® 2.0, Jandel Corporation, San Rafael, CA). Nonparametric statistics were preferred over parametric statistics on transformed data because no single transformation function produced normality and equal variance in all cases. Relative quantities of headspace analytes were compared between loblolly and slash pine at each sampling time using a *t* test or Mann–Whitney Rank Sum test if assumptions were violated (SigmaStat® 2.0). Significance levels for all tests were set at $\alpha = 0.05$.

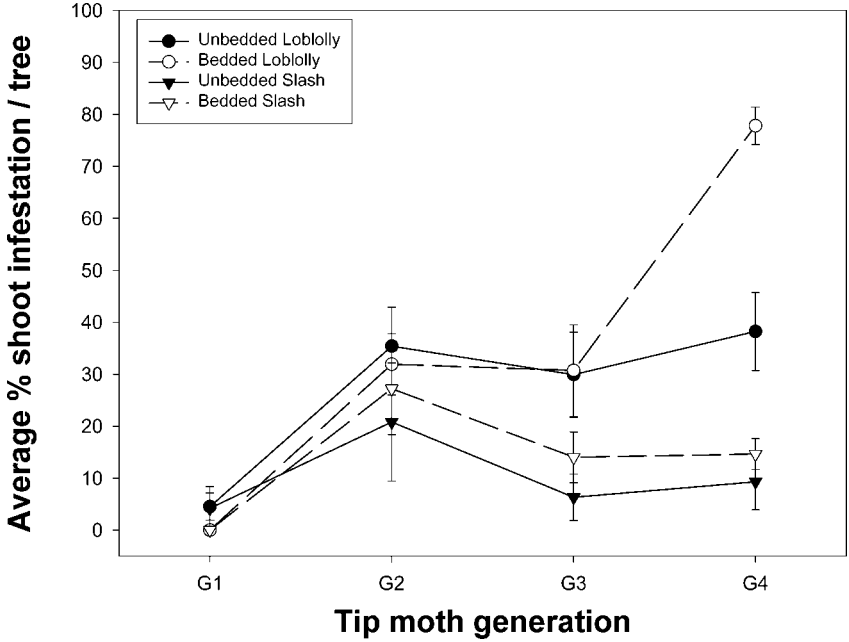

FIG. 1. Average (±SE) top-whorl percent shoot infestation of slash and loblolly pine by *Rhyacionia frustrana* on two separate plots near Rincon, GA, during four tip moth generations (G1-4).

RESULTS AND DISCUSSION

All pupae collected from shoots were identified as *R. frustrana*, so the observed damage was attributed to this species. Damage estimates for slash and loblolly pine were not significantly different at either plot during generations 1 ($H = 6.07$; df $= 3$; $P = 0.11$) and 2 ($H = 4.12$; df $= 3$; $P = 0.25$), but damage to loblolly pine was higher in generations 3 ($H = 7.93$; df $= 3$; $P = 0.048$) and 4 ($H = 43.28$; df $= 3$; $P < 0.001$) (Figure 1). Damage to loblolly pine was three to four times greater than to slash pine during the fourth generation (Figure 1). Damage to slash pine peaked at 26% during the second generation (Figure 1). These results confirm previous observations that, although loblolly pine is more susceptible to tip moth damage overall, slash pine may be equally susceptible during early stages of seedling establishment. The results likewise suggest that host preferences of ovipositing *R. frustrana* shifted in favor of loblolly pine during the seedlings' first growing season, a phenomenon suggested but not documented previously.

No loss of adsorbed volatile compounds was detected ($P \geq 0.05$) on SPME fibers exposed to standards and stored up to 2 wk. Twenty-five compounds,

TABLE 2. ANTENNAL RESPONSES OF MALE AND FEMALE *R. frustrana* EXPOSED TO A SYNTHETIC MIXTURE OF HOST-ASSOCIATED COMPOUNDS USING COUPLED GAS CHROMATOGRAPHY–ELECTROANTENNOGRAPHIC DETECTION (GC–EAD)

Peak number	Compound	Quantity into GC (ng)[a]	Purity (%)	Supplier[b]	Female EAD response (μV)[c]	Male EAD response (μV)
1	(±)-α-Pinene	85	97	Acros	82 ± 28	73 ± 14
2	(±)-Camphene	63	82	Aldrich	38 ± 10	ns[d]
3	(±)β-Pinene	86	98	Aldrich	64 ± 13	88 ± 13
4	(+)-Sabinene	42	98	Aldrich	28 ± 4	ns
5	Myrcene	40	85	Aldrich	69 ± 13	86 ± 17
6	(–) α-Phellandrene	85	55	Aldrich	40 ± 8	48 ± 6
7	α-Terpinene	42	86	Aldrich	ns	ns
8	(±)-Limonene	84	99	Aldrich	75 ± 29	70 ± 13
9	γ-Terpinene	42	98	Aldrich	44 ± 11	61 ± 9
10	*p*-Cymene	43	98	Aldrich	40 ± 9v	ns
11	Terpinolene	43	96	Aldrich	ns	30 ± 5
12	(±)-Linalool	87	97	Aldrich	120 ± 28	129 ± 16
13	(±)-Camphor	98	95	Fluka	87 ± 23	111 ± 18
14	(–)-Bornyl acetate	37	91	Aldrich	ns	ns
15	(±)-Terpinen-4-ol	93	96	Aldrich	104 ± 26	129 ± 15
16	(–)-β-Caryophyllene	90	91	Aldrich	50 ± 10	48 ± 12
17	(–)-Myrtenal	49	96	Aldrich	59 ± 17	75 ± 13
18	4-Allylanisole	48	99	Aldrich	73 ± 18	78 ± 13
19	α-Humulene	44	99	Fluka	31 ± 4	42 ± 9
20	(±)-α-Terpineol	93	99	Aldrich	99 ± 25	103 ± 13
21	(±)-Borneol	97	96	Fluka	77 ± 26	104 ± 17
22	(±)-Verbenone	98	89	Borregaard	68 ±17	115 ± 13

[a] Amount injected into the gas chromatograph of the GC–EAD apparatus. Column effluent was split 1:1, hence antennae were exposed to approximately half this quantity.
[b] Acros Organics, Pittsburgh, PA (Acros); Aldrich Chemical Co., Milwaukee, WI (Aldrich); Fluka Chemical Corp., Milwaukee, WI (Fluka); Borregaard Chemical Co., Sarpsborg, Norway (Borregaard).
[c] Mean ± standard error.
[d] Antennae did not respond to the compound.

primarily monoterpenes and sesquiterpenes, were identified from SPME runs of both slash and loblolly pine, with 12 of these compounds present in only trace amounts (i.e., they did not appear in most runs or never averaged more than 0.5% of the total volatile profile throughout the study) (Table 1). Previous tests confirmed that none of the compounds identified as host volatiles were present as impurities from the fibers or polyethylene bags (data not shown). The relatively large number of compounds detected compared with previous research using Porapak® Q (Ross et al., 1995) suggests that SPME is a more sensitive method for sampling volatiles.

Significant differences were found between the total volatile profiles of slash and loblolly pine for all generations. For simplicity we have shown volatile profiles

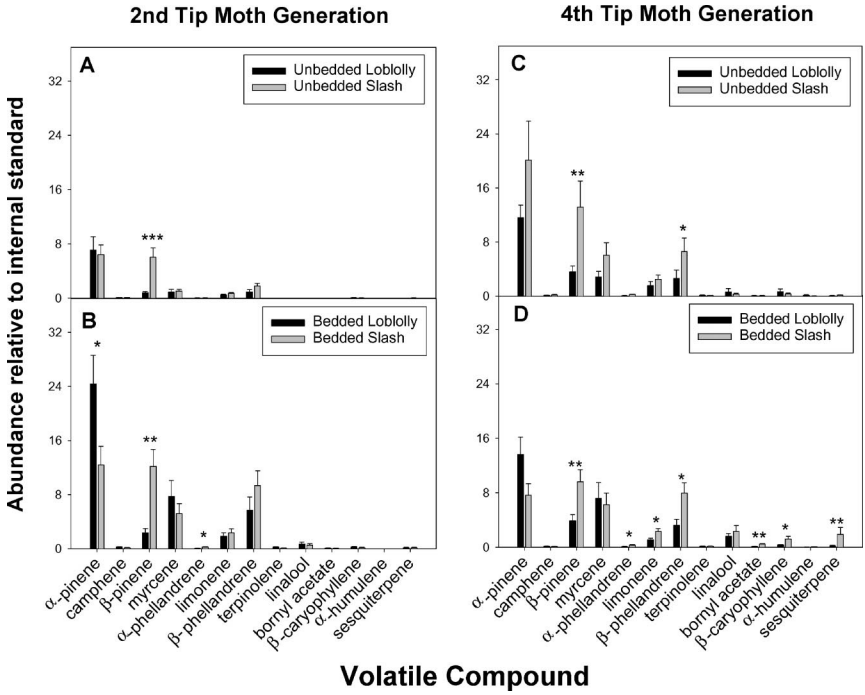

FIG. 2. Relative differences (average ± SE) in headspace composition between slash and loblolly pine during the second (A and B) and fourth (C and D) *Rhyacionia frustrana* generation on unbedded (A and C) and bedded (B and D) plots near Rincon, GA. Compounds showing a significant difference between slash and loblolly pine are indicated by a single ($P < 0.05$), double ($P < 0.01$), or triple ($P < 0.001$) asterisk.

collected during the second and fourth tip moth generations (Figure 2), because these two generations appeared to best represent the change in *R. frustrana* host preference that occurred during the first growing season. Specifically, the second generation caused moderate and similar damage levels in both pine species, whereas damage levels, and presumably *R. frustrana* oviposition preference, were higher for loblolly seedlings by the fourth generation (Figure 1).

Greater differences in volatiles between slash and loblolly pine emerged by the fourth generation (Figure 2a–d). Among the 25 compounds collected (Table 1), 12 (sabinene, camphor, *p*-cymene, myrtenal, 4-allylanisole, α-terpinene, γ-terpinene, terpinen-4-ol, *trans*-verbenol, α-terpineol, borneol, verbenone) were found in trace amounts and did not differ between slash and loblolly pine within either plot ($P > 0.1$) from one generation to the next (data not shown). Barring the possibility of temporal variation in the enantiomeric composition of chiral

members of this group (which our study did not examine), our data suggest that these 12 compounds likely do not play a role in host discrimination by gravid female *R. frustrana*.

At each plot, a number of volatile compounds, which were not present or detected in trace amounts during the second generation, became apparent or increased significantly during the fourth generation (Figure 2). Because damage diverged on the two host species after the second generation, we would also expect compounds acting as host selection cues to diverge quantitatively during this time. Six compounds were detected that quantitatively distinguished slash and loblolly pine during the fourth but not the second generation in the bedded plots (α-phellandrene, limonene, β-phellandrene, bornyl acetate, β-caryophyllene, and an unidentified sesquiterpene) (Figure 2b and d). However, only one of these compounds (β-phellandrene) distinguished the two tree species in both the bedded and unbedded plots (Figure 2a–d).

Although no interspecific differences in relative amounts of myrcene were discovered, as in Ross et al. (1995), these data do support earlier studies showing that β-pinene is present in greater amounts in slash pine compared to loblolly pine. However, β-pinene did not fluctuate significantly in either host during the study. Because the chirality of β-pinene produced by each tree species was not determined, the possible role of β-pinene in host discrimination remains unclear.

The intensity of site preparation and corresponding growth rate of tree seedlings may have had an important effect on volatile emissions, because volatiles from trees on the bedded plot yielded a greater variety of compounds or showed greater differences between slash and loblolly pine. Weeds overtopped the pines on the unbedded plot but never became well established in the bedded area. By October 23, the trees on the unbedded plot averaged only 38.7 and 34.2 cm in height for loblolly and slash pine, respectively, whereas on the bedded site, they averaged 98.7 and 64.4 cm in height, respectively. However, the experiment was not originally designed to test differences in site preparation and, without replication, any conclusions regarding the effects of site preparation on production of volatiles are speculative.

Nineteen different compounds associated with host seedlings elicited responses from antennae of female *R. frustrana* (Figure 3, Table 2). Antennae of males generally exhibited a similar pattern of olfactory sensitivities as females, responding to 17 of 22 tested compounds. In addition, a significant antennal response was detected at the retention time of β-phellandrene, which was present as a contaminant in the synthetic test mixture. Our GC–EAD data indicate that *R. frustrana* are capable of sensing the majority of compounds in the blends of volatiles associated with host seedlings. The extent to which *R. frustrana* can distinguish these compounds or their enantiomers from one another is unknown; nonetheless our data suggest that a large number of compounds could potentially mediate host selection by female *R. frustrana*.

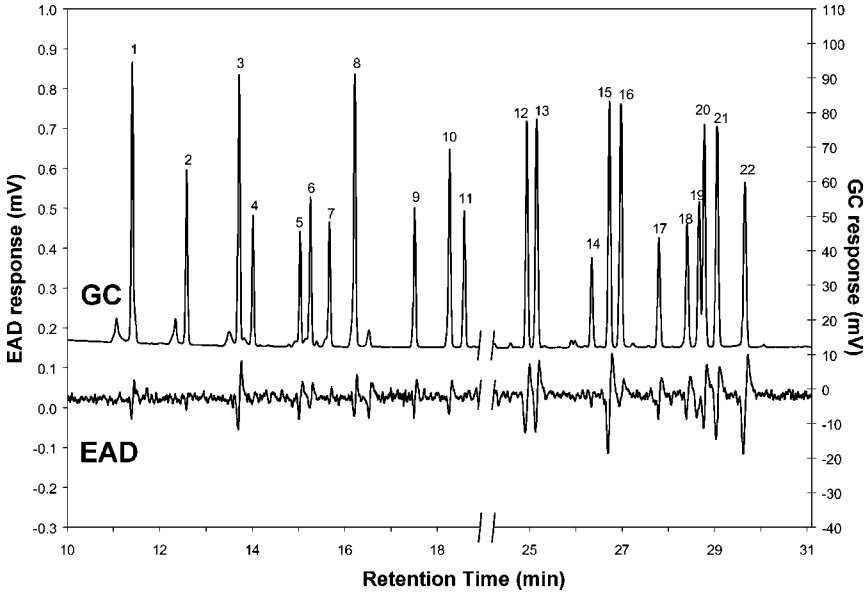

FIG. 3. Simultaneously recorded gas chromatographic (GC) and electroantennographic detection (EAD) traces from a single female *R. frustrana* antenna in response to a synthetic mixture of compounds found associated with foliage of potential host trees, *P. taeda* and *P. elliottii*. Identities of individual GC peaks are listed in Table 2. GC trace represents output from a flame ionization detector.

Location of host plants by phytophagous insects may be mediated by plant volatiles (Städler, 1974; Miller and Strickler, 1984; Hanula et al., 1985; Metcalf, 1987), whereas compounds on the plant surface may mediate oviposition preferences (Städler, 1986; Woodhead and Chapman, 1986; Ross et al., 1995). However, Honda (1995) cites accumulating evidence that plant volatiles may also mediate oviposition by female moths. Previous electrophysiological studies have demonstrated the ability of tortricid moths to detect and distinguish a large number of host-associated odors (Den Otter et al., 1978; Van der Pers, 1981; Rotundo and Tremblay, 1993). In addition, behavioral studies have shown that moth species from a diversity of insect families respond to a broad variety of host-associated compounds, including terpenes (Pivnick et al., 1994; Suckling et al., 1996; Shu et al., 1997; Raguso and Light, 1998; Burguiere et al., 2001). Suckling et al. (1996) stated that EAD was a poor predictor of oviposition-related attraction or inhibition in the light brown apple moth, *Epiphyas postvittana* (Lepidoptera: Tortricidae), a highly polyphagous species. However, they suggested that this approach might work better on monophagous species.

Whereas β-phellandrene was the only compound that met basic criteria for being a host selection cue, its modest correlation with damage levels suggests that this compound is not the only cue mediating host discrimination. The large number of volatile compounds detectable by *R. frustrana* suggests that tip moth oviposition preferences may be mediated by the perception of blends of compounds in specific proportions and not the absolute concentrations of individual semiochemicals. Furthermore, there is a strong possibility that *R. frustrana* can distinguish between the enantiomers of chiral compounds. Therefore, differences in the enantiomeric composition of phytochemicals between these two hosts may influence oviposition preference. Future studies should include enantiomeric analysis of host volatiles and EAD responses of *R. frustrana* to these enantiomers. In addition, the potential importance of visual cues in host selection and oviposition by *R. frustrana* and the possible interactions of visual with chemical cues should be addressed.

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POTENTIAL FOR USE OF SYNTHETIC SEX PHEROMONE FOR MATING DISRUPTION OF THE OLIVE PYRALID MOTH, *Euzophera pinguis*

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Abstract—The potential for pheromone-based mating disruption of the olive pyralid moth (OPM), *Euzophera pinguis*, in olive groves was investigated during the second flight period in small-plot trials in 2002. The female of this species emits a blend of (9*Z*,12*E*)-tetradecadien-1-ol and (9*Z*,12*E*)-tetradecadienyl acetate, which were synthesized for field tests. Mating disruption efficacy in 0.8-ha trials was evaluated using two parameters: reduction of male capture in pheromone traps and reduction of infestation in infestation-prone sites. White rubber septa containing 10 mg of pheromone blend as disruptant were applied at a density of 50 septa/ha for each treatment. Mean catches of *E. pinguis* males in pheromone traps were greatly reduced (> 95%) in pheromone-treated plots relative to similar traps placed in control plots. In addition, significant reductions were recorded (35–40%) in the oviposition and infestation levels during pheromone treatment. The total amount of pheromone blend released from disruption dispensers during the field trials was estimated to average 5.4 mg/ha/day, over 56 days.

Key Words—Mating disruption, olive pyralid moth, *Euzophera pinguis*, Lepidoptera, Pyralidae, (9*Z*,12*E*)-tetradecadien-1-ol, (9*Z*,12*E*)-tetradecadienyl acetate.

INTRODUCTION

The olive pyralid moth (OPM), *Euzophera pinguis* Haworth (Lepidoptera: Pyralidae), is a serious pest of olive crops in Spain, Portugal, and some North African countries. Olive pyralid moth larvae feed on the cambium of the olive

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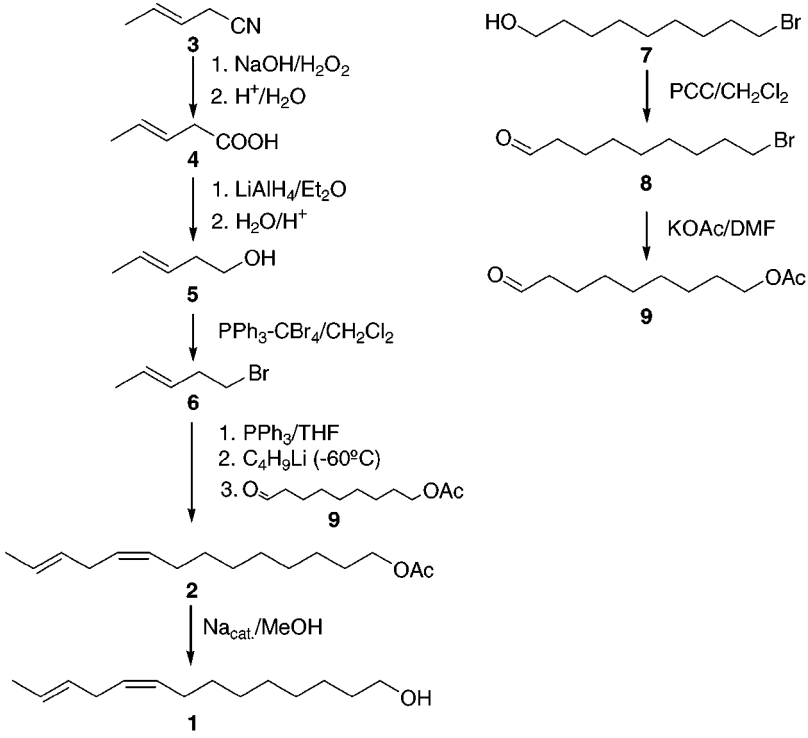
tree, and as a result of larval feeding, tree vigor is heavily diminished. The insect overwinters beneath the bark as a larva, and the first adults emerge in late March, continuing their first flight period until mid-June (Touzeau, 1965). After the first oviposition period, a second flight period occurs in late August to early October. In late spring, mated females lay their eggs singly or in small groups in crevices (Arambourg, 1986) and frass accumulations. Neonate larvae bore quickly into the stem, and any open wounds that expose the cambium are prone to infestation. The highest infestations occur in olive trees that have experienced wounding, especially where mechanical harvesting is used. Because of this endophytic behavior, it is difficult to control the pest using conventional chemical methods, and the application of insecticides against larvae has proven to be ineffective.

The female of this species emits a two-component pheromone blend consisting of (9*Z*,12*E*)-tetradecadien-1-ol [(9*Z*,12*E*)-14:OH)] and (9*Z*,12*E*)-tetradecadienyl acetate [(9*Z*,12*E*)-14:OAc)] (Ortiz et al., unpublished). Traps baited with 1 mg of a 20:1 blend of (9*Z*,12*E*)-14:OH and (9*Z*,12*E*)-14:OAc captured large numbers of males and remained attractive for at least 6 wk (Ortiz et al., 2002). The identification of these sex pheromone components may allow the development of pheromone-based mating disruption as part of an integrated pest management strategy. Mating disruption has been used successfully against several species of pyralid moths, such as *Chilo suppressalis* Wlk. (Casagrande, 1993) and *Scirpophaga incertulas* Wlk. (Cork et al., 1996). However, the success of this approach depends on many factors including the pheromone release device, the population dynamics of the target species (Cardé and Minks, 1995), the number and type of dispensers (Sauer and Karg, 1998), and the efficacy of the formulation (Thorpe et al., 1999). Minks and Cardé (1988) reviewed the evidence indicating that natural pheromone blends are the most effective disruptants. Although pheromone traps are used for monitoring other olive pests and have received considerable research attention (Kelly and Mazomenos, 1992), few studies have been conducted on mating disruption of olive pests. For example, for *Prays oleae*, a synthetic pheromone was found to disrupt the male moths' ability to locate pheromone point sources in olive groves (Mazomenos et al., 1999).

This paper describes syntheses of the olive pyralid moth pheromone components, and initial investigations of the potential for using pheromone-based mating disruption to control this moth in olive groves using small-plot trials, as a preliminary to embarking on a large-scale commercial evaluation.

METHODS AND MATERIALS

General Chemicals and Analytical Procedures. Diethyl ether and THF were dried before use. *E*3-Pentenenitrile (95% purity), pyridinium chlorochromate (PCC), $LiAlH_4$, CBr_4, and 1-bromo-9-nonanol were obtained from Sigma–Aldrich, Germany. Flash chromatography was performed with 200–400



SCHEME 1. Synthesis of (9*Z*,12*E*)-14:OH (**1**) and (9*Z*,12*E*)-14:OAc (**2**).

mesh silica gel. IR spectra were recorded on a THERMO IR-200 instrument. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker spectrometer at 300 MHz in $CDCl_3$. GC analysis was performed on a Varian 3350 instrument with a DB-5 column (30 m $\times$ 0.32 mm, J&W Scientific) programmed from 50°C/1 min, 10°C/min to 250°C using He carrier gas (1.1 ml/min). An SP-2340 column (30 m $\times$ 0.32 mm, Supelco) run with the above program was used to determine isomeric purity. For identification purposes, commercially available (9*Z*,12*E*)-14:OAc (Bedoukian Research, Inc., Danbury, CT) was used. Electron impact mass spectrometry (70 eV) was carried out with an Hewlett-Packard (H-P) 5890 II Plus GC equipped with an HP-5 column (30 m $\times$ 0.32 mm) operated with the above program and coupled to an H-P 5889-B mass spectrometer.

Syntheses. For field experiments, samples of (9*Z*,12*E*)-14:OH **1** and (9*Z*,12*E*)-14:OAc **2** were prepared as shown in Scheme 1.

(E)-3-Pentenoic Acid (***4***). Thirteen milliliters of 30% H_2O_2 (125 mmol) were added dropwise over 20 min to a stirred solution of 5 ml of (*E*)-3-pentenenitrile **3** (3.94

g, 48.6 mmol) and 41.6 ml of 3 N aqueous NaOH (125 mmol). The reaction mixture was heated at 80°C for 2 hr, then for 1 hr at room temperature. The solution was extracted once with diethyl ether (20 ml), which was discarded. The aqueous solution was cooled to 0°C, and the pH was adjusted to 4–5 with 6 N HCl. The suspension was extracted with diethyl ether (4 × 15 ml), and the combined organic phase was washed with brine (2 × 15 ml), dried ($MgSO_4$), filtered, and concentrated to give 3.45 g (71%) of **4**. ^{1}H NMR: 1.65 (m, 3H), 3.01 (d, $J = 6.29$ Hz, 2H), 5.45–5.64 (m, 2H), 11.6 (s, 1H). ^{13}C NMR: 17.7, 37.6, 121.9, 129.8, 178.8. IR ν_{max} (cm^{-1}, film): 3450–2600 (b), 3032 (m), 2977, 1713 (s), 1407 (m), 1291 (m), 1223 (m), 967 (m). MS *m*/*z* (%): 100 (M^+, 34), 82 (10), 55 (100), 45 (16), 41 (34), 39 (39).

(*E*)-*3-Penten-1-ol* (***5***). A solution of acid **4** (1.5 g, 15 mmol) in diethyl ether (20 ml) was added dropwise over 20 min to a stirred and ice-cooled suspension of $LiAlH_4$ (0.77 g, 20 mmol) in diethyl ether (20 ml). The mixture was stirred 1 hr at room temperature. Water was carefully added, and the mixture was poured into ice-diluted H_2SO_4. The solution was extracted with ether (4 × 15 ml), the ether extracts were washed with water and brine, dried over $MgSO_4$, and concentrated *in vacuo* to yield 1.14 g (82%) of **5**. ^{1}H NMR: 1.69 (m, 3H), 1.85 (s, 1H), 2.25 (q, $J = 6.41$ Hz, 2H), 3.62 (t, $J = 6.31$ Hz, 2H), 5.36–5.63 (m, 2H). ^{13}C NMR: 18.0, 36.0, 62.0, 127.1, 128.5. IR ν_{max} (film): 3350 (s), 3040 (m), 2855 (s), 1437 (m), 996 (m). MS *m*/*z* (%): 86 (M^+, 12), 68 (92), 55 (100), 41 (40), 31 (38).

1-Bromo-(E)-3-pentene (***6***). A solution of triphenylphosphine (5.61 g, 21.4 mmol) in dry CH_2Cl_2 was added dropwise to a cooled solution of CBr_4 (3.5 g, 10.7 mmol) in 30 ml of dry CH_2Cl_2. The mixture was stirred for 30 min at 0°C, then a solution of **5** (923 mg, 10.7 mmol) in CH_2Cl_2 was added in small aliquots. The mixture was stirred 8 hr at room temperature, then filtered through a pad of florisil (600 mesh) rinsing with the minimum amount of diethyl ether. The organic layer was concentrated until a white precipitate appeared. Hexane was added, and the solid was filtered off. The filtrate was concentrated and chilled to below –20°C to precipitate any remaining triphenylphosphine oxide. The solvent was removed by rotary evaporation, and the product was obtained as a yellow oil (0.72 g, 45%). ^{1}H NMR: 1.65 (m, 3H), 2.54 (m, 2H), 3.36 (t, $J = 6.31$ Hz, 2H), 5.35–5.63 (m, 2H). ^{13}C NMR: 17.9, 32.9, 36.0, 127.6, 128.3. IR ν_{max} (film): 3062 (m), 2855 (s), 1437 (m), 996 (s). MS *m*/*z* (%): 148/150 (1/1, M^+, 8), 93/95 (1/1, 7), 80/82 (1/1, 5), 69 (100), 55 (46), 41 (99).

9-Bromononanal (***8***). 9-Bromo-nonan-1-ol **7** (1.15 g, 5.1 mmol) was added in small portions to a well-stirred solution of PCC (1.58 g, 7.6 mmol) in CH_2Cl_2 (75 ml). The mixture was stirred at room temperature for 2 hr. Diethyl ether (50 ml) was added, and the supernatant was filtered through a pad of florisil rinsing with 2 × 50 ml diethyl ether. The filtrate was concentrated yielding 1.12 g (98%) of aldehyde **8** as yellow oil. ^{1}H NMR ($CDCl_3$): 1.20–1.38 (m, 10H),

1.56 (m, 2H), 1.78 (m, 2H), 2.36 (dt, J = 7.26, 1.79 Hz, 2H), 3.37 (t, $J = 6.81$ Hz, 2H), 9.69 (t, $J = 1.65$ Hz, 1H). ^{13}C NMR ($CDCl_3$): 21.2, 28.0, 28.5, 29.1, 32.7, 33.7, 33.9, 43.8, 202.7. IR ν_{max} (film): 2929 (s), 1725 (s), 1462 (m). MS *m*/*z* (%): 221/223 (1/1, 1), 220/222 (1/1, M^+, trace), 176 (28), 178 (24), 97 (71), 81 (56), 69 (37), 55 (100).

9-Oxo-nonyl acetate (***9***). Aldehyde **8** (1.12 g, 5.05 mmol) was added to a suspension of KOAc (991 mg, 10.1 mmol) in DMF (8 ml), the mixture was stirred 2 hr at 120°C, then poured into water (100 ml) and extracted with diethyl ether (3 × 50 ml). The combined extracts were washed several times with water until no DMF was detected by GC. The ether extract was concentrated under reduced pressure yielding **9** (960 mg, 95%) as a colorless oil. ^{1}H NMR ($CDCl_3$): 1.22–1.26 (m, 8H), 1.58–1.61 (m, 4H), 2.02 (s, 3H), 2.41 (dt, $J = 7.28$, 1.84 Hz, 2H), 4.03 (t, $J = 6.72$ Hz, 2H), 9.75 (t, $J = 1.65$ Hz, 1H). ^{13}C NMR: 21.0, 22.0, 25.8, 28.5, 29.0, 29.0, 29.2, 43.8, 64.5, 171.2, 202.8. IR ν_{max} (film): 2931 (s), 1738 (s), 1726(s), 1465, 1242. MS *m*/*z* (%): 157(5), 141 (7), 123 (12), 97 (70), 43 (100).

(9*Z*,12*E*)*-14:OAc* (***2***). Triphenylphosphine (1.26 g, 4.8 mmol) was added over 10 min to a vigorously stirred solution of bromide **6** (720 mg, 4.8 mmol) in 25 ml of THF under Ar atmosphere, and the reaction mixture was stirred 8 hr at room temperature. Then, butyllithium (2.4 ml, 2.0 M in hexane) was carefully added over 5 min, the mixture was stirred 30 min, cooled to –60°C, and treated with aldehyde **9** (912 mg, 4.8 mmol) in 20 ml of dry THF, and again stirred at –60°C under Ar for 2 hr. The reaction mixture was stirred overnight at room temperature, then diluted with hexane (30 ml), and filtered to remove most of the Ph_3PO. Residual Ph_3PO was removed by precipitation, by chilling a hexane solution of the crude product and filtration, and the filtrate was concentrated. GC analysis of the residue on an SP-2340 column resolved the *ZE* and *EE* isomers (Z*E*/*EE* ratio, 13:1). The *ZE* isomer identification was confirmed with an authentic standard. The mixture of isomers was purified by flash chromatography (2.5 × 25 cm) on silica gel impregnated with 10% $AgNO_3$, eluting with a stepwise gradient of 10–50% ethyl acetate in hexane to give 738 mg (61%, >97% isomeric purity) of acetate **2**. The NMR spectral data closely matched literature values (Matveeva et al., 1988; Sharma et al., 1989).^{1}HNMR: 1.25–1.37 (m, 10H), 1.57–1.78 (m, 5H), 2.02 (m, 2H), 2.05 (s, 3H), 2.72 (t, $J = 4.4$ Hz, 2H), 4.05 (t, $J = 6.77$ Hz, 2H) 5.31–5.49 (m, 4H). ^{13}C NMR: 17.9, 21.0, 25.9, 27.0, 28.6, 29.1, 29.2, 29.4, 29.6, 30.4, 64.6, 125.1, 127.6, 129.6, 130.4, 171.3. IR ν_{max} (film): 3010 (m), 2928 (s), 1743 (s), 1240 (s), 966 (m), 723 (m). MS *m*/*z* (%): 252 (M^+, 3), 192 (28), 135 (17), 121 (28), 110 (25), 95 (48), 81 (95), 79 (88), 67 (100), 55 (67), 43 (97), 41 (51), 39 (18).

(9*Z*, 12*E*)*-14:OH* (***1***). A small piece of Na was added to 650 mg of acetate **2** (2.57 mmol) in MeOH (20 ml). The reaction mixture was stirred 2 hr at room temperature and then quenched with saturated aqueous NH_4Cl (25 ml) and

extracted with ether (4 × 20 ml). The ether extract was washed with brine, dried over $MgSO_4$, and the solvent was removed to give 530 mg (98%) of alcohol **1**. ^{1}H NMR: 1.35 (m, 12H), 1.56 (m, 1H), 1.65 (m, 3H), 2.02 (m, 2H), 2.72 (m, 2H), 3.63 (t, $J = 6.64$ Hz, 2H), 5.31–5.49 (m, 4H). ^{13}C NMR: 17.9, 25.8, 27.1, 29.2, 29.4, 29.5, 29.7, 30.5, 32.8, 63.1, 125.1, 127.7, 129.7, 130.4. IR ν_{max} (film): 3339 (s), 3010 (m), 2926 (s), 1451 (m), 965 (m), 723 (m). MS *m*/*z* (%): 210 (M^+, 1), 192 (2), 121 (14), 110 (12), 95 (34), 81 (70), 68 (100), 55 (55), 41 (52), 39 (18).

Field Tests. The main experimental site in the Baeza area (North Andalucia, Spain) was a 3.5-ha olive grove and an adjacent plot of 0.4 ha (800 m away) that served as a control. The two experimental blocks were approximately 0.8 ha (15 rows × 12 trees) in size and separated by at least 500 m, the rows being in line with the usual wind direction. The trees (Picual variety) were about 25-yr-old, 3–4 m in height, and planted on a 7 × 7 m spacing. Experimental olive groves were infested (detected by monitoring with pheromone-baited traps) with OPM and were not sprayed with insecticides during the study. The preliminary monitoring results obtained in the experimental area during the first *E. pinguis* flight period (early April to late June), showed that mean moth catches were 20–30/moths/trap/night.

Field tests were started before the beginning of the second flight period of *E. pinguis* and concluded after the flight was over, between August 20 and October 15, 2002.

Chemicals, Dispensers, and Formulations. The pheromone blend used for *E. pinguis* disruption was a mixture of (9*Z*,12*E*)-14:OH (> 95% chemical purity, > 97% isomeric purity) and (9*Z*,12*E*)-14:OAc (> 95% chemical purity, > 97% isomeric purity) in a 20:1 ratio, which was used to monitor this moth. Dilutions were made in hexane (HPLC grade) to give 50 mg/ml stock solutions, then aliquots (2 × 100 μl) were loaded onto white rubber septa (9–10 mm OD, Aldrich, #Z10072-2), to give the desired dose of 10 mg per septum. This dose was based on a previous monitoring test (Ortiz, unpublished) in which catches in funnel traps baited with 5 mg lures were drastically lower than catches in traps baited with 1 mg.

Dispenser Placement. Forty septa were deployed in each plot ($N = 2$), at a rate of 50 ha^{-1} (500 mg pheromone/ha) up to 1 wk before the second flight period started (end of August). Dispensers were attached to branches using a wire in the upper third of the tree canopy.

Field Monitoring. Trap data from the control plots were used to determine the duration and time of the peak period of male flight activity. Funnel traps were baited with 1 mg of (9*Z*,12*E*)-14:OH and (9*Z*,12*E*)-14:OAc at a 20:1 ratio on white rubber septa and contained a DDVP strip to kill captured moths. Traps ($N = 2$) were placed in the center of the two pheromone-treated plots and the control plot and were checked every week. During the field study, rubber septum lures were replaced 6 wk after they had been installed.

Estimated Release Rates of Pheromone. To determine the amount of pheromone released from the septa in the field, groups of five septa were attached to a tree 1 km away from the experimental areas. Septa were removed periodically, and stored in sealed glass vials at –10°C until analysis. Control septa stored at –10°C were analyzed for comparison. Groups ($N = 3$) of five septa were extracted for 8 hr in hexane with a Soxhlet extractor. The extracts were analyzed by gas chromatography (GC) on a Varian 3350 GC equipped with a DB-5 capillary column (splitless, 30 m × 0.32 mm ID; J&W Scientific) programmed from 50°C/0 min, 50°C/min to 100°C for 6 min, then at 20°C/min to 250°C. The mean amount of (9*Z*,12*E*)-14:OH remaining was calculated using calibration curves prepared with authentic samples (Bedoukian Research, Inc., Danbury, CT) as external standard. Although (9*Z*,12*E*)-14:OAc was detected in each sample, the amount could not be determined accurately because of the small initial load and the presence of impurities in the sample after extraction.

Evaluation of Mating Disruption. The parameters used for assessing disruption efficacy were (i) reduction of male catches in funnel traps baited with synthetic pheromone, and (ii) the numbers of wounds infested in the pheromone-treated plots and the control plot. Disruption of pheromone source location was assessed by comparing the numbers of males captured in traps. Two funnel traps were placed in each replicate of the pheromone-treated field and the control field. Each trap was baited with a white rubber septum loaded with 1 mg of a 20:1 blend of (9*Z*,12*E*)-14:OH and (9*Z*,12*E*)-14:OAc, and each trap contained a DDVP strip to kill captured moths. Trap catches were counted every week, and traps were randomly repositioned in the block after inspection. The percentage of communication disruption, %CD, was calculated according to the following equation: $\%CD = 100(N_c - N_t)/N_c$, where N_c and N_t are the number of males caught in control and test traps, respectively.

The second method to assess the levels of disruption consisted of assessing oviposition into incisions made with a knife. The incisions (N = 20/plot) were horizontal (1 × 3 cm wide), and deep enough to cut through the vascular tissues, causing local sap retention, on a group of trunks randomly distributed across the plot, thus simulating a set of pruning wounds. Olive pyralid moth infestations were estimated within each replicate of the pheromone-treated field ($N = 2$) and control field by counting the presence of eggs or frass accumulation in the 20 incisions/plot. Counts were taken on a weekly basis. Infestation level percentages were compared by χ^2 test. The SPSS 9.0 statistical package was used for analysis.

RESULTS

Trap monitoring showed that during the second flight period captures in the disruptant-treated plots were drastically lower compared to catches in the traps located outside the treated areas, on all collection dates at each test site (Figure 1),

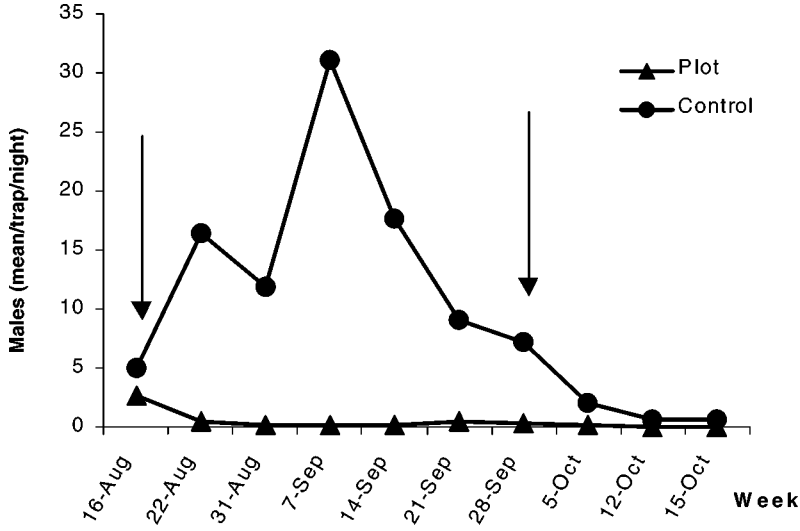


FIG. 1. Numbers of male moths caught in pheromone-baited traps in 0.8 ha treated and control plots. Arrows indicate replacement of trap baits.

suggesting that the orientation of males to the pheromone traps was disrupted by the 20:1 blend of (9*Z*,12*E*)-14:OH and (9*Z*,12*E*)-14:OAc. Mean male moth capture in the plots was 71 per trap/wk in the control plots, compared to 3 and 2 per trap/wk in the plots treated with septa, a 97% reduction in trap catch ($P < 0.05$). In both pheromone-treated plots, almost complete suppression of trap catches of *E. pinguis* male moths was maintained over the entire second flight period (Figure 1).

Analysis of the pheromone remaining in the formulation exposed in the field showed that, on average, approximately 40% of the applied pheromone was recovered from septa after 6 wk under field conditions. Thus, the mean amount of (9*Z*,12*E*)-14:OH released from septa dispensers during the field trials was estimated to be ~300 mg/ha over 56 days.

In control plots, 55% of wounds were infested by OPM at the end of the flight period. The pheromone treatment reduced wound infestation by 40% in plot 1 and by 35% in plot 2. The differences in the number of infested wounds in control and pheromone-treated plots were significantly different for both pheromone-treated plots ($P < 0.05$).

DISCUSSION

Almost complete suppression of trap catches of *E. pinguis* male moths was maintained during the test period. The experiments showed that a slow-release

formulation of the pheromone achieved up to 97% communication disruption for at least 8 wk, even with the comparatively low dosages of pheromone used in the study. (9*Z*,12*E*)-14:OAc is a component of the pheromone blend of many pyralid species (Phelan and Baker, 1990), and a few studies have been performed on mating disruption of other pyralids (e.g., Ryne et al., 2001). Although rubber septa have been used previously for mating disruption (Bengtsson et al., 1994), this type of dispenser has an approximately first order release rate, which decreases exponentially as dispensers age. Nevertheless, with septum dispensers, complete or nearly complete trap shutdown was consistently achieved, even when low levels of pheromone were used (e.g., Ohtani et al., 2001). Our results also demonstrate the potential of the mating disruption technique in preventing males from finding pheromone-baited traps. However, these results do not necessarily translate into reduced mating (Cardé and Minks, 1995).

Methods to assess mating activity include use of pheromone-baited sticky traps (e.g., McBrien et al., 1998), or more reliably, by crop damage estimates (e.g., Mazomenos et al., 1999). Because of the unknowns regarding OPM biology, we chose infestation as the better parameter to assess mating activity. The pheromone-treated plots had lower infestation levels than the control field. The significant reduction in oviposition and infestation was particularly encouraging in view of the relatively low amounts of pheromone used in these preliminary, small-plot trials.

In summary, field tests performed during the second flight period in 2002 showed that application of an *E. pinguis* pheromone blend has the potential to disrupt mating in this species, and may reduce new infestations caused by their progeny. However, many aspects of the biology and behavior of this pyralid need to be understood in order to further develop the mating disruption technique. Furthermore, practical details such as optimal pheromone dose and formulation, optimal dispenser type and placement, and dispenser longevity need to be worked out and extended to large-scale plots. Nevertheless, this study has demonstrated the potential for using pheromone-based mating disruption to control this species.

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THE EFFECT OF EXOGENOUS JASMONIC ACID ON INDUCED RESISTANCE AND PRODUCTIVITY IN AMARANTH (*Amaranthus hypochondriacus*) IS INFLUENCED BY ENVIRONMENTAL CONDITIONS

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Abstract—*Amaranthus hypochondriacus* is a C_4 pseudocereal crop capable of producing reasonable grain yields in adverse environmental conditions that limit cereal performance. It accumulates trypsin inhibitors and α-amylase inhibitors in seeds and leaves that are considered to act as insect feeding deterrents. Foliar trypsin and α-amylase inhibitors also accumulate by treatment with exogenous jasmonic acid (JA) in controlled laboratory conditions. Three field experiments were performed in successive years to test if two nonphytotoxic dosages of JA were capable of inducing inhibitor activity in *A. hypochondriacus* in agronomical settings, and if this induced response reduced insect herbivory and insect abundance in foliage and seed heads. The performance of JA-treated plants was compared to insecticide-treated plants and untreated controls. The effect of exogenous JA on the foliar levels of six additional putatively defence proteins was also evaluated. Possible adverse effects of JA induction on productivity were evaluated by measuring grain yield, seed protein content, and germination efficiency. The results present a complex pattern and were not consistent from year to year. To some extent, the yearly variability observed could have been consequence of growth under drought versus nondrought conditions. In a drought year, JA-treated plants had lower levels of insect herbivory-derived damage in apical leaves and panicle than control plants, whereas in nondrought years, there was an inconsistent effect on aphids, with no effect on lepidopteran larvae. JA

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treatments reduced the size of the insect community in seed heads. The effect varied with year. Exogenous JA did not adversely affect productivity, and in the absence of drought stress, the higher dosage enhanced grain yield. Induction of defensive proteins by JA, although sporadic, was more effective in nondrought conditions. The patterns of foliar protein accumulation observed suggest that they may be part of a constitutive, rather than inducible, chemical defense mechanism that is developmentally regulated and critically dependent on the environment. The results emphasize the difficulties that are often encountered when evaluating the performance of chemical elicitors of induced resistance in field settings.

Key Words—*Amaranthus hypochondriacus*, defense proteins, drought stress, field experiments, induced resistance, insect community, jasmonic acid, productivity.

INTRODUCTION

Amaranthus species are C_4 dicotyledonous pseudocereals. They are cultivated as leaf vegetables or as grain-producing crops (Teutonico and Knorr, 1985; Kauffman and Weber, 1990). Amaranth leaves are a good source of vitamins and minerals, whereas seeds have relatively high protein contents of superior quality due to their elevated percentage of lysine and sulphur amino acids (Downton, 1973; Segura-Nieto et al., 1992). The high nutritional quality of its seed proteins, which can supplement cereal and legume proteins, and the high rates of growth allowing up to six generations per year, make amaranth an attractive alternative crop for commercial application (Rawate, 1983). In addition, many amaranth species, including *Amaranthus hypochondriacus* L., can produce reasonable yields in poor soils and/or semiarid conditions characterized by low water availability, high light intensity and temperature (Dean, 1986; A. Borodanenko, personal communication). The cultivation of *A. hypochondriacus* in México is widespread, although most of the production concentrates in unirrigated land in the states of Puebla, Tlaxcala, Morelos, and the Federal District (Espitia-Rangel, 1990). Several insect pests affect this crop in the above regions and can significantly reduce yields if not controlled. Control is usually achieved by chemical insecticides (Aragón-García and López-Olguín, 2001). Little is known about defense mechanisms, whether constitutive or inducible, that *A. hypochondriacus* might employ to reduce insect-derived damage. Previous studies reported the isolation and characterization of protease and α-amylase inhibitors in seeds that were proposed to have a defensive role against grain-infesting insects (Valdés-Rodríguez et al., 1993; Chagolla-López et al., 1994). Subsequent studies revealed that protease and α-amylase inhibitors are also present in the leaves of *A. hypochondriacus*. Moreover, these inhibitors further accumulate by foliar application of nontoxic dosages of exogenous jasmonic acid (JA), under controlled conditions of temperature and light (Nagamatsu-López, 2004; Sánchez-Hernández, 2001). This suggests that JA could be used as a chemical elicitor of induced resistance in this crop.

JA and its volatile methyl ester are found in many species, and have been identified as endogenous regulators of wound-induced chemistry in plants and as signal molecules in the responses of plants to herbivory (Reinbothe et al., 1994; Baldwin, 1999; Karban, 1999; Staswick and Lehman, 1999; Thaler, 1999a). The ability of JA to stimulate the expression of proteins putatively involved in plant defense (e.g., polyphenoloxidases, peroxidases, and protease inhibitors), has been associated with an increase of natural plant resistance in field settings. Previous results with tomato, grape vines, lettuce, and carrots indicate that exogenous JA has the potential to be a useful tool for the control of herbivores in the field (Karban, 1999; Thaler, 1999a,b,c; Omer et al., 2000; Thaler et al., 2001). As aptly indicated by Thaler (1999b), the agricultural use of chemical elicitors that have the capacity to reduce insect herbivory, such as JA, should be subjected to a careful cost/benefit analysis, weighing any advantages brought about by induced resistance against possible penalties on productivity, mostly caused by the allocation of plant resources to the synthesis of defense chemicals.

In this investigation, the use of exogenous JA to induce resistance against insect damage in field cultivated *A. hypochondriacus* was evaluated. Three consecutive experiments were performed, and the effects of JA on induced resistance were measured by monitoring changes in the insect community in foliage and seed heads. The effect of JA on the levels of several defense-related proteins in leaves and seed of *A. hypochondricus*, as well as on productivity-related variables, such as yield, seed protein content, and percent germination, was also determined.

METHODS AND MATERIALS

Field Experiments. The study was performed in the experimental fields of the Institute of Agricultural Sciences (ICA) of the University of Guanajuato, México. The fields are situated in the locality of "El Copal," municipality of Irapuato, state of Guanajuato (24°44′44″ North latitude and 101°19′19″ West longitude) at 1745 m above sea-level. The climate is classified as semiwarm subhumid, with average annual temperatures of 17.4–18.8°C and rainfall around 700 mm. The soil is alkaline (pH 8.1) with the first 30 cm layer classified as loam–sand–clay, and the second 30 cm layer as clay–loam–sand. The organic matter content of the soil was 2.32% (first 30 cm layer) and 2.80% (30–60 cm layer). Nitrogen content was 0.056% in both layers, whereas potassium and phosphorus in the first 30 cm layer were 210 ppm and 2.73 meq/100 g, respectively. Amaranth plants (*A. hypochondriacus* var. San Antonio) were grown from seed during the summer and fall of the years 2000–2002. This improved variety was developed by A. Borodanenko (ICA) and is particularly well suited for growth in the semiarid conditions prevalent in this region. The fields were fertilized with 120–60–0 kg/ha (N–P–K) starter fertilizer. In 2001 and 2002, the soil was also mixed with organic compost (2 ton/ha).

Fields were irrigated, before sowing. No additional irrigation was applied, and the experimental plants were dependent on rainfall for subsequent water. Sowing and seedling thinning were performed manually. Plants were spaced at ≈0.2 m intervals and divided into experimental units consisting of 1.56× 5 m plots having three furrows separated by 0.76 m. The field was flanked by two additional rows of plants. Each furrow had an average of 40 plants, giving a density of ≈16 plants/m^2. Fields were weeded manually throughout the experiment. In 2000 and 2001, the experimental plots were organized into blocks in which 16 different treatments were randomly distributed. Each treatment was replicated four times: (1) untreated control; (2) low JA (0.5 mM JA; ≈0.545 μmoles JA per plant); (3) high JA (1.5 mM JA; ≈1.635 μmoles JA per plant); and (4) chemical insecticide (125 ml/100 l; Rogor™; Agricultura Nacional, México). Twelve treatments consisted of a single application of low JA, high JA, or insecticide at either one of four different phenological stages. Three treatments consisted of the repeated application of low JA, high JA, or insecticide in the four stages. The phenological stages selected were young plants (15 d after seedling emergence and thinning (det)), developing plants (35 det), adult flowering plants (60 det), and senescing plants in the process of grain filling (90 det). The JA dosage applied was based on previous reports (Thaler et al., 1996; Thaler, 1999a) and on preliminary laboratory results that showed induction of trypsin and α-amylase inhibitor activities in leaves of young *A. hypochondriacus* plants sprayed with similar nonphytotoxic concentrations of JA (Nagamatsu-López, 2001; Sánchez-Hernández, 2001). JA was produced from the alkaline hydrolysis of methyl jasmonate (Sigma Chemical Co., St. Louis, MO), as described by Farmer et al. (1992). JA was dissolved in 1 ml of acetone and subsequently dispersed in an appropriate volume of distilled water containing 0.1% (v/v) of a nonionic surfactant (INEX-A® ; Cosmocel S.A., México) and buffered to ≈pH 5.0 with a pH and water hardness regulator (Buffex® ; Cosmocel S.A., México). The final volume of the solutions was adjusted throughout the experiments to ensure that different-sized plants received the same JA dosage. The chemical insecticide was similarly dispersed in identical volumes of water plus additives. Plants were sprayed with a backpack sprayer. A separate sprayer was used for each treatment. Neighboring plants were shielded from the spray with a large piece of plastic supported by wood poles. Spraying was always performed in the morning, between 8 and 10 A.M., to minimize evaporation and dispersion by wind. Leaf samples from five plants per experimental plot were collected 48 hr after the first three applications (15, 35, and 60 det); plants treated 90 det were not sampled since they already showed signs of senescence. Sampling was performed by cutting four leaves per plant from the upper third section of the plant, at different distances from the apex. These younger leaves were less damaged than older leaves. While on the field, leaf samples were temporally stored in plastic ice boxes filled with solid CO_2. Once in the laboratory, they were stored at –80˚C, ground with liquid nitrogen, and lyophilized. Lyophilized samples were utilized for biochemical assays (see below).

Effect on Insect Herbivory and the Insect Community in Foliage and Seed Heads. In 2000, folivory by lepidopteran caterpillars, mostly by *Spodoptera* sp., was determined. This was performed by visually inspecting the damage produced to the apical end of the plants, including the youngest leaves and the nascent and developing panicule. Sampling was performed thrice, on the 4th and 27th day of August and on the 9th day of September. In the first sampling, 60 plants per treatment, from one replicate only, were examined, whereas in the latter two, 60 plants per treatment in all four replicates were examined, giving a total of 2160 observations. Damage was scored according to a scale involving four different levels: (I) low damage (0–10% of plant area damaged); (II) moderate damage (10–25% of plant area damaged), (III) extensive damage (25–50% of plant area damaged); and (IV) severe damage ($>$50% of plant area damaged).

To sample the insect community in seed heads, five seed heads per experimental plot were collected 2–3 wk after the last spaying (90 det), giving a total of 20 seed heads per treatment per year. The sampling procedure was performed by quickly covering each randomly chosen grain head with a large transparent plastic bag, which was subsequently severed from the rest of the plant by bending at its base. This was done in order to trap the insects present in the seed heads during the seed maturation stage. The bags were frozen at -20°C (year 2000) or stored in a well-ventilated room after their treatment with tablets of aluminium phosphide (Agro-Fum^{TM} 57; Centro Agroindustrial S.A., México; year 2001), prior to insect collection, counting, and storage (in 30% (v/v) aqueous ethanol solutions). Grain was harvested and cleaned with an air stream (mid November and early December). Yields of grain per hectare were extrapolated from yields produced by the experimental 7.8 m^2 plots. Samples of grain were stored at 4°C until required for biochemical and protein content analyses and germination assays (see below).

A third field experiment, following a similar design, was performed in 2002. In this experiment, only the high dosage of JA (1.5 mM) was repeatedly applied, at approximately the same four developmental stages, and was compared to insecticide-treated and control plants. No leaf or seed samples were taken for biochemical assays and productivity measurements, nor was yield determined. However, plants were sampled five times to determine the abundance of lepidopteran caterpillars and aphids infesting the foliage. Sampling was performed 1 wk after each of the first four sprayings. The final sampling was performed 2 wk after the last spraying. Because of the low level of lepidopteran caterpillar infestation in 2002, all experimental plants were examined on each sampling date, and an additional sixth sampling was performed close to the end of the growing season. All sampled caterpillars were taken alive to the laboratory where they were monitored for parasitism or allowed to develop into adults to facilitate identification (results not shown). Sampling for aphid abundance in foliage was performed by thoroughly searching the abaxial surface of all leaves of four plants per experimental plot. A total number of 16 plants per treatment per sampling date were examined. Aphid

specimens were also taken to the laboratory for identification. For insect counts in seeds heads, 12 seed heads per experimental plot were sampled, as above. A higher number of samples per plot was taken to compensate for the fact that only two replicates were sampled this year. A total of 24 seed heads per treatment were sampled. Insects were aspirated directly from the plastic bags used to trap them by means of portable insect Vacs (BioQuip®, Gardena, CA). They were subsequently frozen at -20°C and placed in 30% ethanol solutions prior to identification. Identification was done by M.D. Salas, at the Entomology Laboratory of the Institute for Agricultural Sciences (ICA) of the University of Guanajuato, México. For analysis, the insect community in seed heads was divided into phloem feeding (PF) insects, chewing (CH) insects, and predaceous and parasitoid (PP) insects.

Meteorological Conditions. Experiments were performed from late spring to late autumn of the years 2000, 2001, and 2002. Seeds were sown in the 2nd week of June (year 2000) and 4th week of June (years 2001 and 2002). Total monthly rainfall, average insolation and evaporation per month, and maximum, mean, and minimum monthly values for relative humidity and ambient temperature were recorded by a weather station manned by the ICA of the University of Guanajuato (Appendix). The station is situated less than 1 km from the experimental fields.

Enzyme and Inhibitor Assays. Crude extracts were prepared by mixing lyophilized leaf samples or ground seed flour samples in appropriate buffers (see below). Leaf extracts were employed to measure the levels of activity of the following putative defense-related proteins: trypsin inhibitors, chymotrypsin inhibitors, α-amylase inhibitors, polyphenol oxidases, peroxidases, leucine aminopepetidases, β-1,3-glucanases, chitinases, and polygalacturonases. Seed extracts were used to measure trypsin, chymotrypsin, and α-amylase inhibitor activity. Trypsin and chymotrypsin inhibitor levels of activity (TIA and CTIA) were determined according to Erlanger et al. (1961) and Gervaix et al. (1991), using $N\alpha$-benzoyl-L-arginine-*p*-nitroanilide hypochloride (BApNA) and *N*-benzoyl-L-tyrosine-*p*-nitroanilide (BTpNA) as substrates, respectively. Alpha amylase inhibitor activity (AAIA) was measured according to Bird and Hopkins (1954), using starch as substrate. TIA, CTIA, and AAIA were determined as inhibitor units per milligram of dry weight. Leucine aminopeptidase activity (LAPA) was determined according to Appel (1974), using leucine-*p*-nitroanilide as substrate. Polyphenol oxidase (PPO) and peroxidase (PRX) activities were measured according to Thaler et al. (1996), using caffeic acid and guaiacol as substrates, respectively. Polygalacturonase activity (PGA) was assayed according to Gross (1982), based on the measurement of reducing sugars released from the hydrolysis of polygalacturonic acid. Chitinase activity (CHIA) was determined according to Villagómez-Castro et al. (1992), using 4-methylumbelliferyl-β-D-$N,N^{\mathrm{I}},N^{\mathrm{II}}$-triacetylchitotrioside hydrate as substrate. β-1,3-Glucanase activity (BGA) was assayed according to Zheng and Wozniak (1997) using laminarin as substrate. All activity assays, except the chitinase assay, were modified to fit a microplate format. The activity of foliar proteins

(except enzyme inhibitors) was calculated per milligram total protein. Protein content was measured according to the Bradford method (Bradford, 1976), employing a commercial kit (Bio-Rad Laboratories, USA). The enzyme controls employed were trypsin and chymotrypsin from bovine pancreas, laminarinase from *Trichoderma* sp., peroxidase from horseradish, tyrosinase from mushroom, polygalacturonase from *Aspergillus japonicus*, and chitinase from *Streptomyces griseus* (all from Sigma-Aldrich Chemical Co., USA). Alpha-amylases and leucine aminopeptidases were extracted from larvae of the red flour beetle (*Tribolium castaneum* Herbst) and the large grain borer (*Prostephanus truncatus* Horn), respectively (Sandoval-Cardoso, 1991; Chagolla-López et al., 1994). All enzyme substrates employed were also from Sigma-Aldrich.

Protein and Germination Assays. Seed protein content was determined by multiplying total seed nitrogen content by a 5.85 conversion factor (Becker et al., 1981). Seed nitrogen content was determined using the Kjeldahl method (AOAC, 2000). To evaluate percent germination, 100 seeds per treatment per replicate were surface sterilized by washing in a 1% solution of sodium hypochlorite for 10 min. The seeds were subsequently rinsed with running water to eliminate excess hypochlorite and placed on humidified filter paper contained in sterile petri dishes. Seeds were incubated in darkness at 28°C for 2 d. Germination was scored by counting the number of germinated seeds, and they were classified into five groups based on seedling length (0–1, 1–2, 2–3, 3–4, and 4–5 cm, respectively) in order to determine seed vigor. All assays were repeated at least thrice.

Statistical Analysis. Herbivory by noctuid larvae in 2000 was analyzed to determine if the degree of damage was independent of the treatment applied (null hyphothesis). To test this, data were cross-tabulated, and the chi-square (χ^2) test was applied to the resulting table (Sokal and Rohlf, 1969). To understand further the relation between level of damage and treatment, the cross-tabulated data were analyzed taking into account all possible pairs of treatments, resulting in each case in a 2×4 contingency table, to which the χ^2 test was applied. To test for differences in aphid and lepidopteran larvae counts in foliage in 2002, a general lineal model, assuming a Poisson distribution for the number of insects, was fitted using treatment (for aphids and larvae) and sampling date (for aphids) as explanatory variables. Data were analyzed to test for independence of treatment and sampling date on the insect counts obtained. For aphid counts in each sampling date, all pairs of treatments were contrasted and subjected to analyses of deviance. For insect counts in seed heads, an analysis of deviance was performed independently for each insect community. Treatment, Year, and Treatment $\times$ Year interaction were used as explanatory variables. In each Year and for each insect community, all pairs of treatments were contrasted and subjected to analyses of deviance. In each case, χ^2 tests were applied to test for independence between insect counts and treatment.

Chemical and plant productivity variables were examined by analyses of variance (ANOVA). For seed chemistry and plant productivity variables, the overall

effect of treatment, year, and its interaction were analyzed. For foliar chemistry, the overall effect of treatment, year, and development stage, as well as their interactions were analyzed. A separate ANOVA was performed per year to test the effect of treatment on seed chemistry and productivity variables, and an additional ANOVA was performed per year to test the effect of treatment on foliar chemistry at each development stage.

The values of the F statistic, as well as the probability of that value under the null hypothesis of equality of treatments, are reported in the results. For ANOVA tables where the F test was significant at 0.05 or lower, the Tukey method was used to obtain 95% simultaneous confidence intervals for the differences among treatment means. All data were analyzed by using the S-plus statistical package (S-PLUS Professional Edition, Version 6.0.2 Release 1 for Microsoft Windows).

RESULTS

In 2000 and 2001, the complete field experiments consisted of 16 different treatments; in 12 of these JA, low (0.5 mM) or high (1.5 mM), or insecticide were applied only once in each of four phenological stages. The effects observed did not differ much from those obtained from plants in which sprayings were applied repeatedly in all four stages. Therefore, for clarity, only those treatments in which JA or insecticide were applied continuously are included here. A simplified experiment was performed in 2002; only the high JA dosage was used, which was applied four times throughout the growing season, and the effects were likewise compared to those obtained in insecticide-treated and untreated controls.

Effect of Exogenous JA on Herbivory by Noctuid Caterpillars. Plants were sampled three times through August and September 2000 to test for caterpillar damage, in the apical section of the plant, which included the youngest leaves and the emerging and developing panicles. The sampling period coincided with the onset and development of flowering and with a high population density of noctuid larvae. A χ^2 test applied to the cross-tabulated data derived from the 2160 observations (Table 1) was highly significant ($\chi^2 = 25.42$, $P = 0.002$), indicating that the levels of damage were dependent on treatment. A χ^2 test, taking into account all possible pairs of treatments (Table 2), indicated that the dependence observed between damage level and treatment could be attributed to the heterogeneity of effect between untreated controls (more damaged) and the insecticide and JA treatments (less damaged). No significant difference in herbivory was found between insecticide- and JA-treated plants, nor between the two dosages of JA employed.

Effect on Abundance of Insect Herbivores in Foliage. The number of lepidopteran larvae and aphids infesting the foliage of *A. hypochondriacus* plants was monitored in 2002. In contrast to the two previous years, the level of infestation by

TABLE 1. NUMBER OF *A. hypochondriacus* PLANTS CROSS-CLASSIFIED BY LEVEL OF DAMAGE BY LEPIDOPTERAN LARVAE[a] AND TREATMENT[b] (YEAR 2000)

	Level of damage				
Treatment	Low (0–10%)[c]	Moderate (10–25%)[c]	Extensive (25–50%)[c]	Severe (>50%)[c]	Total
Control	453	36	38	13	540
JAL	479	35	15	11	540
JAH	487	28	20	5	540
INS	489	25	14	12	540
Total	1908	124	87	41	2160

[a] Damage produced by lepidopteran larvae was evaluated in apical leaves and panicles.
[b] Cultivated plants were untreated (control) or treated with insecticide (INS) or with 0.5 mM (JAL) or 1.5 mM jasmonic acid (JAH).
[c] Percent area damaged.

lepidopteran larvae was low. Consequently, six independent samplings of all experimental plants yielded a total of 18, 14, and 10 specimens in untreated controls, JA-treated, and insecticide-treated plants, respectively. Considering the scarcity of lepidopteran insects, all larval counts were combined and analyzed as one sample. The difference in larval abundance between treatments was not statistically different ($\chi^2 = 0.00$, $P = 0.313$). Conversely, aphid numbers in foliage were significantly affected by treatment (T) ($\chi^2 = 345.5$, $P < 0.001$), sampling date (S) ($\chi^2 = 313.82$, $P < 0.001$), and T $\times$ S interaction ($\chi^2 = 243.53$, $P < 0.001$). Highly significant differences were detected when aphid counts were analyzed per sampling date (Table 3, Figure 1). JA treatment significantly reduced aphid numbers only on the first sampling date (08/13/2002), whereas aphid counts were higher than controls on all other sampling dates, except the last one (10/8/2002). Curiously, insecticide treatment did not reduce aphid numbers below levels found

TABLE 2. CHI-SQUARE TEST FOR INDEPENDENCE OF ALL TREATMENT PAIRS ON DEGREE OF DAMAGE PRODUCED BY LEPIDOPTERAN LARVAE IN *A. hypochondriacus* PLANTS (YEAR 2000)[a]

Treatment contrast	χ^2	P
Control vs. INS*	14.47	0.002
Control vs. JAL*	10.88	0.012
Control vs. JAH*	11.37	0.001
INS vs. JAL	1.85	0.605
INS vs. JAH	4.12	0.249
JAL vs. JAH	3.00	0.283

[a] Data from Table 1 were used for the analysis.
*Significant differences at $P < 0.05$

TABLE 3. ANALYSIS OF DEVIANCE OF CONTRASTS AMONG TREATMENT PAIRS ON APHID ABUNDANCE PER SAMPLING DATE IN FOLIAGE OF UNTREATED *A. hypochondriacus* PLANTS (C) OR PLANTS TREATED WITH INSECTICIDE (INS) OR A HIGH (1.5 MM; JAH) DOSAGE OF JASMONIC ACID

Sampling date	Treatment contrast	df	Deviance residual	df	Residual deviance	*P*
08/13/2002	Null			7	51.62	0.002
	C vs. JAH	1	9.33	6	42.29	
	Null			7	39.26	0.536
	C vs. INS	1	0.38	6	38.87	
	Null			7	19.20	0.015
	INS vs. JAH	1	5.97	6	13.22	
08/27/2002	Null			7	47.91	<0.001
	C vs. JAH	1	18.29	6	29.62	
	Null			7	52.34	0.021
	C vs. INS	1	5.31	6	47.03	
	Null			7	57.61	0.042
	INS vs. JAH	1	4.14	6	54.46	
09/10/2002	Null			7	55.60	<0.001
	C vs. JAH	1	13.41	6	42.20	
	Null			7	58.87	0.746
	C vs. INS	1	0.11	6	58.76	
	Null			7	50.49	<0.001
	INS vs. JAH	1	11.2	6	39.29	
09/24/2002	Null			7	23.60	0.050
	C vs. JAH	1	3.86	6	19.74	
	Null			7	54.76	<0.001
	C vs. INS	1	30.61	6	24.15	
	Null			7	28.28	<0.001
	INS vs. JAH	1	13.15	6	15.13	
10/08/2002	Null			7	20.49	0.893
	C vs. JAH	1	0.018	6	20.47	
	Null			7	36.89	0.021
	C vs. INS	1	5.33	6	31.56	
	Null			7	25.19	0.015
	INS vs. JAH	1	5.96	6	19.23	

in controls and clearly promoted aphid abundance towards the end of the sampling period (Table 3, Figure 1).

Effect on the Insect Community in Seed Heads. A total of 38 different insect species were identified in seed heads of *A. hypochondriacus* sampled in three consecutive growing seasons. They were identified at least to family, and to species when possible. For the analysis of the community in seed heads, all insects were further divided into three groups: phloem feeding (PF), chewing (CH), and predaceous and parasitoid (PP) insects (Table 4). An analysis of deviance indicated that the abundance of the three communities in seed heads was dependent on

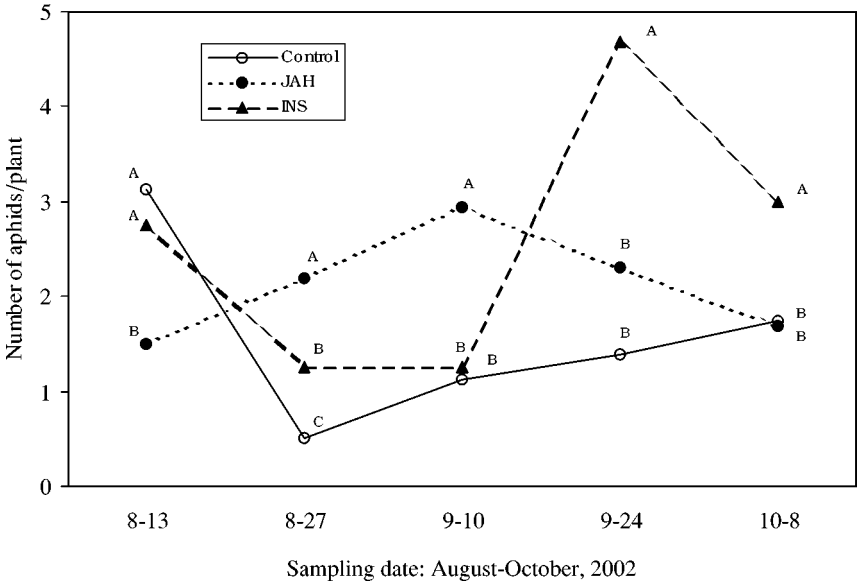


FIG. 1. Aphid abundance in foliage of untreated, field grown *A. hypochondriacus* plants (control) and plants treated with 1.5 mM jasmonic acid (JAH) or insecticide (INS). Different letters above the symbols in each sampling date represent statistically different aphid counts (at $P < 0.05$), obtained after applying a χ^2 test to all contrasted pairs of treatments (see Table 3).

the effect of time (T), year (Y), and its interaction (Table 5). PF and PP insects were most abundant in 2000, whereas CH insects were in 2001. An analysis of deviance of contrasts among all treatment pairs (Table 6) revealed that in 2000, JA-treated plants had the lowest counts of PF insects, which were significantly different from those found in untreated controls and insecticide-treated plants. In 2000, the effect of JA dosage was also significant, with low JA-treated plants having the smallest PF insect counts. This behavior was partially repeated in 2002, year in which high JA-treated plants had lower PF insect counts than controls (but higher than insecticide-treated plants). In contrast, no significant differences in PF insect counts between control plants and JA-treated plants (high and low) were detected in 2001 (Table 6, Figure 2a). CH insect counts were lower than controls in JA-treated plants only in 2000; in 2001 and 2002, no significant differences between controls and JA-treated plants were detected (Table 6, Figure 2b). With respect to PP insects, low JA-treated plants had lower counts than controls and high JA-treated plants in 2000. Similar to PF and CH insects, PP insect counts in seed heads of JA-treated plants were not different from untreated controls in 2001, whereas in 2002, high JA-treated plants had lower PP counts than controls (Table 6,

TABLE 4. INSECT SPECIES COLLECTED IN SEED HEADS OF *Amaranthus hypochondriacus* DURING THREE CONSECUTIVE FIELD EXPERIMENTS (2000 TO 2002) IN IRAPUATO, GTO., MEXICO

	Year		
	2000	2001	2002
Phloem feeding insects			
Hemiptera			
Miridae			
Lygus lineolaris (Palisot de Beauvois)	237	240	95
Litomiris debilis (Uhler)			21
Pentatomidae			
Euschistus sp.	37	61	
Holcosthethus sp.	54		
Brochymena arborea (say)	23		
Undetermined		32	
Coreidae			
Ceraleptus sp.	133	108	33
Catorhintha sp.		94	
Thyreocoridae			
Galgupha sp.	63		
Corimelaena sp.	20	21	
Lygaeidae			
Oedancala sp.	1372	98	
Tingidae			
Physatocheila sp.	34		83
Undetermined			16
Largidae			
Largus succinctus (L.)		4	
Homoptera			
Membracidae			
Micrutalis sp. + *M. malleifera* (Fowler)	181	140	45
Cicadellidae			
Undetermined	13		
Chewing Insects			
Coleoptera			
Cleridae			
Undetermined	20		
Chrysomelidae			
Diabrotica balteata (Le Conte)		24	19
D. undecinpunctata (Mannerheim)		9	
Epitrix cucumeris (Harris)			8
Disonycha sp.	21	10	
Gastrophysa sp.			32
Bruchidae			
Acanthoscelides obtectus (Say)	19	3	
Phalacridae			
Undetermined		5	

TABLE 4. CONTINUED

	Year		
	2000	2001	2002
Tenebrionidae			
Undetermined		68	
Dermoptera			
Forficulidae			
Doru taenatium (Dorhn)	18	125	35
Lepidoptera			
Pyralidae			
Undetermined	20		
Hymenoptera			
Eurytomidae			
Harmolita tritici (Fitch)			13
Predaceous and parasitoid insects			
Hemiptera			
Anthocoridae			
Orius insidiosus (say)	12		49
Pentatomidae			
Podisus maculiventris (say)	19		
Saldidae			
Undetermined	77		
Coleoptera			
Coccinelidae			
Hippodamia convergens (Guerin)	13		19
Epilachna tredecimnotata (Latreille)		2	
Scymnus sp.	197		
Carabidae			
Lebia viridis (Say)	34	15	
Lampiridae			
Undetermined		3	
Hymenoptera			
Eulophidae			
Undetermined			4
Formicidae			
Formica sp.			13

Figure 2c). The results also indicate that the PP component of the seed head insect community in *A. hypochondriacus* was particularly sensitive to insecticide treatment (Figure 2c).

Effect on Seed and Leaf Chemistry and Plant Productivity. Seed TIA and AAIA levels and yield were affected by the overall effect of T, Y, and its interaction. CTIA was affected by T and Y, whereas the effect of Y was significant for seed protein content and germination efficiency (Table 7). The effect of T was subsequently analyzed separately each year. The results are summarized in

TABLE 5. ANALYSIS OF DEVIANCE OF THE EFFECT OF TREATMENT, YEAR, AND ITS INTERACTION ON THE INSECT COMMUNITY IN SEED HEADS OF UNTREATED *A. hypochondriacus* PLANTS[a]

Variable	Factor	df	Deviance residual	df	Residual deviance	*P*
Phloem feeders	Null			43	4755.21	
	Treatment (T)	3	1102.51	40	3652.69	<0.001
	Year (Y)	2	1483.06	38	2169.63	<0.001
	T×Y	5	404.50	33	1765.14	<0.001
Chewing insects	Null			43	280.56	
	Treatment (T)	3	32.04	40	248.51	<0.001
	Year (Y)	2	67.87	38	180.64	<0.001
	T×Y	5	15.28	33	165.35	0.009
Predators	Null			43	529.69	
and parasitoids	Treatment (T)	3	69.10	40	460.58	<0.001
	Year (Y)	2	788.21	38	172.27	<0.001
	T×Y	5	10.62	33	161.55	0.059

[a] The analysis includes data from three consecutive field experiments (2000 to 2002).

Table 7 and Figure 3. In 2000, JA-treated plants had higher levels of TIA (high JA, Figure 3b) and AAIA (low JA, Figure 3d) than untreated control plants. On the other hand, JA-treated plants (low JA) produced lower yields than insecticide-treated plants (Figure 3a). In 2001, differences were obtained only in plants treated with high JA: these produced higher grain yields than insecticide-treated plants (Figure 3a) and higher levels of AAIA than low JA-treated and control plants (Figure 3d). The highly significant effect that Y had on seed chemistry and plant productivity variables was evident in the differences detected between both years. Thus, in 2001, overall seed protein content was reduced 20%, seed yield was doubled, seed germination efficiency increased from 64.4 to 83.7%, TIA and AAIA levels were reduced 3- and 1.4-fold, respectively, and CTIA levels increased 5.3-fold.

The ANOVA shown in Table 8 indicates that most foliar protein activity levels were affected by Y, development stage (DS), and their interaction. The exceptions were LAPA, which was only affected by Y and Y × DS interaction, and PRX and PPO activities, which were not detected at all (results not shown). The overall effect of T was significant for CTIA, BGA, and CHIA, whereas the levels of TIA, BGA, and CHIA were affected by the T × DS interaction. Only BGA and AAIA levels were affected by the T × Y × DS interaction.

The effects of treatment on foliar chemistry were also analyzed separately per year (Table 9 and Figure 4). They indicate that in 2000, exogenous JA treatments produced few significant differences in foliar protein activity levels, which were never detected in young plants. Thus, the only significant changes detected in JA-treated plants, with respect to untreated controls and/or insecticide-treated plants,

TABLE 6. ANALYSIS OF DEVIANCE OF CONTRASTS AMONG TREATMENT PAIRS ON THE INSECT COMMUNITY IN SEED HEADS OF UNTREATED *A. hypochondriacus* PLANTS (C) OR PLANTS TREATED WITH INSECTICIDE (INS) OR WITH LOW (0.5 mM; JAL) OR HIGH (1.5 mM; JAH) DOSAGES OF JASMONIC ACID

		Year 2000				Year 2001				Year 2002			
Variable	Treatment contrast	df	Deviance residual	df	Residual deviance	*P*	df	Deviance residual	df	Residual deviance	*P*	df	Deviance residual	df	Residual deviance	*P*
Phloem feeders	Null			7	2357.44	<0.001			7	71.56	<0.001			7	90.24	<0.001
	C vs. INS	1	758.13	6	1599.31		1	28.14	6	43.42		1	64.99	6	25.25	
	Null			7	2229.79	<0.001			7	53.17	0.226			7	41.00	<0.001
	C vs. JAH	1	865.89	6	1363.9		1	1.46	6	51.70		1	11.45	6	29.55	
	Null			7	2405.61	<0.001			7	27.16	0.547					
	C vs. JAL	1	1009.00	6	1396.51		1	0.36	6	26.79				n.d.		
	Null			7	255.47	0.038			7	74.52	<0.001			7	34.64	<0.001
	INS vs. JAH	1	4.32	6	251.15		1	16.84	6	57.78		1	22.75	6	11.88	
	Null			7	305.93	<0.001			7	67.70	<0.001					
	INS vs. JAL	1	22.17	6	283.76		1	34.83	6	32.87				n.d.		
	Null			7	55.3	0.008			7	44.44	0.070					
	JAH vs. JAL	1	6.96	6	48.55		1	3.29	6	41.16				n.d.		
Chewing insects	Null			7	20.44	0.162			7	88.22				7	28.52	<0.001
	C vs. INS	1	1.95	6	18.49		1	18.11	6	70.11	<0.001	1	15.93	6	12.58	
	Null			7	28.23	0.009			7	86.83				7	26.73	0.753
	C vs. JAH	1	6.91	6	21.37		1	0.75	6	86.08	0.386	1	0.10	6	26.63	
	Null			7	13.91	0.009			7	76.06						
	C vs. JAL	1	6.91	6	7.01		1	2.37	6	73.70	0.124			n.d.		
	Null			7	33.02	0.215			7	36.41				7	31.01	<0.001
	INS vs. JAH	1	1.54	6	31.48		1	11.57	6	24.85	<0.001	1	13.59	6	17.42	
	Null			7	18.65	0.215			7	19.95						
	INS vs. JAL	1	1.55	6	17.11		1	7.48	6	12.47	0.006			n.d.		

TABLE 6. CONTINUED

Variable	Treatment contrast	Year 2000					Year 2001					Year 2002				
		df	Deviance residual	df	Residual deviance	*P*	df	Deviance residual	df	Residual deviance	*P*	df	Deviance residual	df	Residual deviance	*P*
	Null			7	20	1.000			7	28.89						
	JAH vs. JAL	1	0.00	6	20		1	0.05	6	28.44	0.502			n.d.		
Predators	Null			7	99.08	<0.001			7	8.88	0.160			7	45.14	<0.001
and parasitoids	C vs. INS	1	29.06	6	70.02		1	1.97	6	6.90		1	35.66	6	9.47	
	Null			7	80.4	0.593			7	2.88	0.295			7	17.31	0.017
	C vs. JAH	1	0.29	6	80.12		1	1.10	6	1.78		1	5.69	6	11.62	
	Null			7	48.31	<0.001			7	3.68	0.819					
	C vs. JAL	1	17.38	6	30.93		1	0.05	6	3.63				n.d.		
	Null			7	129.06	<0.001			7	13.95	0.015			7	19.91	<0.001
	INS vs. JAH	1	23.68	6	105.57		1	5.88	6	8.06		1	13.68	6	6.23	
	Null			7	57.75	0.213			7	12.56	0.103					
	INS vs. JAL	1	1.55	6	56.19		1	2.33	6	9.91				n.d.		
	Null			7	79.54	<0.001			7	5.46	0.413					
	JAH vs. JAL	1	13.25	6	66.29		1	0.67	6	4.79				n.d.		

Note. n.d. = not determined.

were in AAIA (Figure 4c), and in PGA (Figure 4e) in developing plants (35 det), and in CHIA (Figure 4g) in mature plants (60 det). All other differences detected involved control and insecticide-treated plants (Figure 4a, b, and g).

In 2001, the number of differences detected in JA-treated plants increased slightly. In contrast to the previous year, most differences were observed in young and developing plants. Accordingly, significant changes in JA-treated plants, with respect to controls and/or insecticide-treated plants, were detected in CHIA in young plants (Figure 4f), AAIA and BGA in young and mature plants (Figure 4c and g), and TIA and CTIA in developing plants (Figure 4a and b).

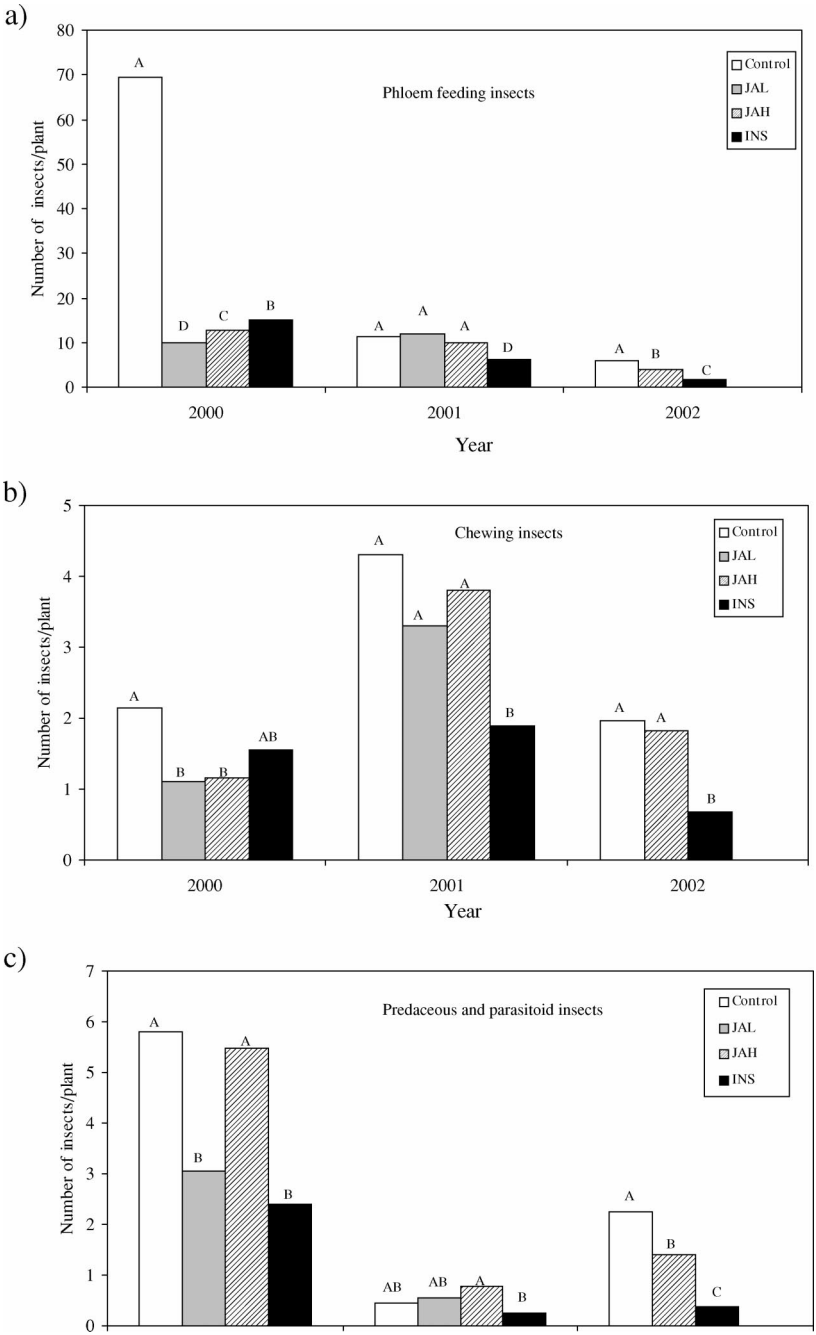
The effects of Y, DS, and Y $\times$ DS interaction on foliar protein activity are seen in Figure 4. Except for PGA levels (Figure 4e) that increased concomitantly with development to reach a maximum point in mature plants in both years, all other foliar protein activities showed variations between year and development stage, having in some cases (e.g., LAPA and CTIA) completely opposite patterns of accumulation during development.

The most pronounced changes in overall foliar protein activity levels detected between years involved a 4.8-fold reduction in TIA levels in mature plants in 2001, and 11.7- and 4.1-fold increases in CHIA levels in leaves of young and mature plants, respectively.

DISCUSSION

The results present a complex pattern and were not consistent from year to year. Nevertheless, they indicate that exogenous JA (1) significantly affected the insect community in *A. hypochondriacus*, (2) had no negative effect on productivity, and (3) had only a sporadic effect (both enhancing and inhibitory) on foliar and seed protein activities. Furthermore, environmental conditions and ontogeny were significant factors affecting most of the chemical variables analyzed. Thus, it is likely that multiple biotic and abiotic stimuli produced responses on *A. hypochondriacus* plants that were superimposed with those induced by exogenous JA.

In 2000, JA-treated plants had lower levels of damage by noctuid larvae that feed on the young leaves and panicle of the apical portion of *A. hypochondriacus* plants (Tables 1 and 2). JA treatment also reduced the number of PF insects in seed heads in 2 years of the 3-year study, particularly in 2000. CH insects were also negatively affected by JA treatments in 2000 (Table 6, Figure 2a and b). However, the observed negative effect on insects considered to be important pests of *A. hypochondriacus* in the Irapuato area and other amaranth producing regions of México (see Table 4; Salas-Araiza, 1999; Aragón-García and López-Olguín, 2001) was not translated into larger grain yields in 2000 (Figure 3a). Moreover, high JA-treated plants produced higher yields than controls and insecticide-treated plants in 2001, a year in which no effects on PF and CH insects in seed heads were


a)
Phloem feeding insects
Number of insects/plant
Year
2000
2001
2002
Control
JAL
JAH
INS
b)
Chewing insects
Number of insects/plant
Year
c)
Predaceous and parasitoid insects
Number of insects/plant

detected (Table 6, Figure 2a and b). The lack of any benefits, in terms of yield, in *A. hypochondriacus* plants showing an induced resistance response mediated by exogenous JA, suggests that this species is tolerant to insect herbivory and, therefore, that productivity may not be seriously affected by insect-derived damage. In tomato plants, the lack of a positive effect on yield in JA-treated plants showing increased levels of defense-related proteins and lower levels of herbivory and herbivore numbers was also observed. In that case, however, this effect was attributed to nondamaging levels of herbivores during experimentation rather than to an inherent tolerance to herbivory in tomato (Thaler, 1999a,b; Thaler et al., 2001).

The above suggests that JA was able to induce chemical changes in *A. hypochondriacus* that made plants less attractive to lepidopteran larvae and PF and CH insect pests. However, the lack of a consistent induction of any of the defense-related proteins analyzed, especially in 2000, when the negative effect was more pronounced, strongly suggests that other unidentified JA-induced chemicals could have been responsible for the effect observed. Thus, *A. hypochondriacus* might rely, similar to barley and sorghum, on chemical compounds such as alkaloids and phenolics for defense (Corcuera, 1993), which might have accumulated, as has been observed in several other plant species, in response to exogenous JA (Blechert et al., 1995; Memelink et al., 2001). This is in contrast to other plants, such as tomato, in which a causal link between JA-induced accumulation of proteins and a reduction in herbivore performance has been firmly established (Orozco-Cardenas et al., 1993; Felton et al., 1994; Stout et al., 1994; Thaler et al., 1996; Cipollini and Redman, 1999; Thaler 1999a,b).

On the other hand, the results obtained from exogenous JA may be related to the finding that insecticide treatment either reduced, or had a tendency to show the lowest levels in most of the foliar proteins tested. This was probably an indication that insect herbivory was an important inducing factor and that JA treatment was incapable of further increasing levels above those induced by insect damage. Another possible scenario is that the foliar proteins assayed in amaranth were insensitive to exogenous JA due to their constitutive and developmentally regulated pattern of accumulation discussed below.

←

FIG. 2. The abundance of (a) phloem feeding insects, (b) chewing insects, and (c) predaceous and parasitoid insects in seed heads of untreated, field grown *A. hypochondriacus* plants (control) and plants treated with insecticide (INS) or with low (0.5 mM, JAL) or high (1.5 mM, JAH) dosages of jasmonic acid (JA). The results obtained from seed heads sampled in three consecutive field experiments are shown (years 2000–2002). Different letters above the bars represent significantly different counts (at $P < 0.05$), obtained after applying a χ^2 test to all contrasted pairs of treatments (see Table 6).

TABLE 7. ANOVA OF THE EFFECT OF TREATMENT, YEAR, AND ITS INTERACTION ON CHEMICAL AND PRODUCTIVITY RELATED VARIABLES IN SEED OF *A. hypochondriacus* PLANTS SUBJECTED TO FOUR DIFFERENT TREATMENTS (0.5 mM JA, 1.5 mM JA, INSECTICIDE, AND UNTREATED), DURING TWO CONSECUTIVE FIELD EXPERIMENTS (2000 AND 2001)

	TIA			CTIA			AAIA			Yield			Protein content (%)			Germination (%)		
Factor	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Treatment (T)	3	3.19	0.044	3	3.34	0.037	3	5.39	0.006	3	4.16	0.017	3	n.s.		3	n.s.	
Year (Y)	1	191.13	<0.001	1	1910.90	<0.001	1	51.11	<0.001	1	75.63	<0.001	1	215.77	<0.001	1	47.10	<0.001
T×Y	3	6.89	0.002	3	n.s.		3	6.54	0.002	3	6.82	0.002	3	n.s.		3	n.s.	
Residuals	22			23			24			24			24			23		

Note. TIA = trypsin inhibitor activity; CTIA = chymotrypsin inhibitor activity; AAIA = α-amylase inhibitor activity; n.s. = not significant at $P < 0.05$.

TABLE 8. ANOVA OF THE EFFECT OF TREATMENT, YEAR, DEVELOPMENT STAGE AND THEIR INTERACTIONS ON FOLIAR CHEMISTRY IN *A. hypochondriacus* PLANTS SUBJECTED TO FOUR DIFFERENT TREATMENTS (0.5 MM JA, 1.5 MM JA INSECTICIDE, AND UNTREATED), DURING TWO CONSECUTIVE FIELD EXPERIMENTS (2000 AND 2001)

	TIA			CTIA			AAIA			LAPA			PGA			BGA			CHIA		
Factor	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Treatment (T)	3	n.s.		3	4.40	0.007	3	n.s.		3	n.s.		3	n.s.		3	4.68	0.005	3	3.81	0.014
Year (Y)	1	137.15	<0.001	1	199.07	<0.001	1	76.67	<0.001	1	n.s.		1	40.24	<0.001	1	10.45	0.002	1	153.40	<0.001
Development stage (DS)	2	167.31	<0.001	2	61.82	<0.001	2	104.09	<0.001	2	8.92	<0.001	2	224.70	<0.001	2	125.30	<0.001	2	194.10	<0.001
T × Y	3	8.21	<0.001	3	n.s.		3	n.s.		3	n.s.		3	n.s.		3	13.58	<0.001	3	9.22	<0.001
T × DS	6	3.04	0.014	6	n.s.		6	n.s.		6	n.s.		6	n.s.		6	12.84	<0.001	6	4.09	0.002
Y × DS	2	299.72	<0.001	2	63.87	<0.001	2	55.10	<0.001	2	94.08	<0.001	2	13.29	<0.001	2	82.14	<0.001	2	117.16	<0.001
T × Y × DS	6	2.18	0.054	6	n.s.		6	n.s.		6	n.s.		6	n.s.		6	4.17	0.001	6	n.s.	
Residuals	70			64			72			71			55			63			63		

Note. TIA = trypsin inhibitor activity; CTIA = chymotrypsin inhibitor activity; AAIA = α-amylase inhibitor activity; LAPA = leucine aminopeptidase activity; PGA = polygalacturonase activity; BGA = β-1,3-glucanase activity; CHIA = chitinase activity; n.s. = not significant at $p<0.05$.

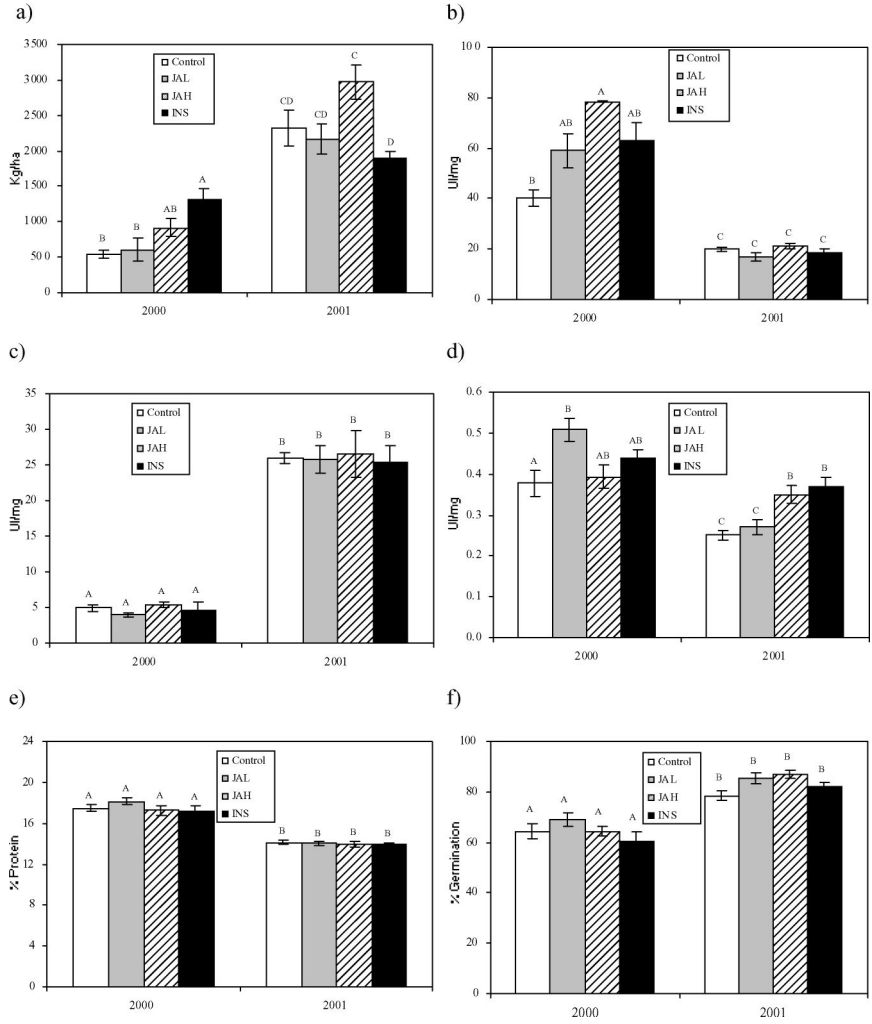


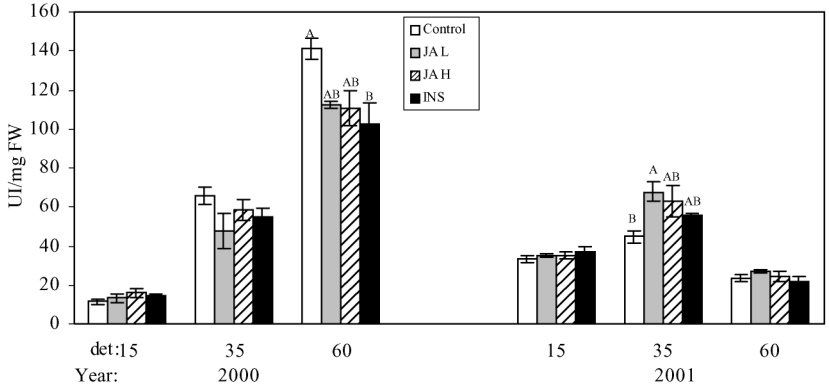
FIG. 3. Mean values (±SE) of grain yield (a), trypsin inhibitor activity levels (b), chymotrypsin inhibitor activity levels (c), α-amylase inhibitor activity levels (d), protein content (e), and percent germination (f), in seed from untreated field grown *A. hypochondriacus* plants (control) or plants treated with a chemical insecticide (INS) or with low (0.5 mM, JAL) or high (1.5 mM, JAH) dosages of jasmonic acid (JA). The results of two independent field experiments performed on consecutive years (2000 and 2001) are shown. Bars with different letters are significantly different at $P < 0.05$ (ANOVA followed by Tukey test).

TABLE 9. ANOVA OF THE EFFECT OF TREATMENT IN DIFFERENT DEVELOPMENTAL STAGES PER YEAR ON CHEMICAL AND PLANT PRODUCTIVITY VARIABLES IN *A. Hypochondriacus* PLANTS SUBJECTED TO FOUR DIFFERENT TREATMENTS (0.5 mM JA, 1.5 mM JA, INSECTICIDE, AND UNTREATED

		Year 2000												Year 2001											
		YL			DL			ML			S			YL			DL			ML			S		
Variable	Factor	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Yield	Treatment		n.d.			n.d.			n.d.		3	6.61	0.007		n.d.			n.d.			n.d.		3	4.66	0.022
	Residuals										12												12		
TIA	Treatment	3	n.s.		3	n.s.		3	4.34	0.030	3	5.92	0.014	3	n.s.		3	3.90	0.037	3	n.s.		3	n.s.	
	Residuals	11			12			11			10			12			12			12			12		
CTIA	Treatment	3	n.s.		3	n.s.		3	3.76	0.041	3	n.s.		3	n.s.		3	16.78	<0.001	3	n.s.		3	n.s.	
	Residuals	4			12			12			11			12			12			12			12		
AAIA	Treatment	3	n.s.		3	4.31	0.028	3	n.s.		3	4.28	0.029	3	4.59	0.023	3	n.s.		3	5.86	0.011	3	9.80	<0.001
	Residuals	12			12			12			12			12			12			12			12		
LAPA	Treatment	3	n.s.		3	n.s.		3	n.s.			n.d.		3	n.s.		3	n.s.		3	n.s.			n.d.	
	Residuals	11			12			12						12			12			12					
PGA	Treatment	3	n.s.		3	6.24	0.038	3	n.s.			n.d.		3	n.s.		3	n.s.		3	n.s.			n.d.	
	Residuals	3			5			12						12			11			12					
BGA	Treatment	3	n.s.		3	4.62	0.025	3	n.s.			n.d.		3	8.96	0.002	3	n.s.		3	19.76	<0.001		n.d.	
	Residuals	11			11			12						12			8			9					
CHIA	Treatment	3	n.s.		3	n.s.		3	25.18	<0.001		n.d.		3	19.68	<0.001	3	n.s.		3	n.s.			n.d.	
	Residuals	8			7			12						12			12			12					

Note. YL = young leaf; DL = developing leaf; ML = mature leaf; S = seed; TIA = trypsin inhibitor activity; CTIA = chymotrypsin inhibitor activity; AAIA = α-amylase inhibitor activity; LAPA = leucine aminopeptidase activity; PGA = polygalacturonase activity; BGA = β-1,3-glucanase activity; CHIA = chitinase activity; n.d. = not determined; n.s. = not significant at $P < 0.05$.

a)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JA L
JA H
INS
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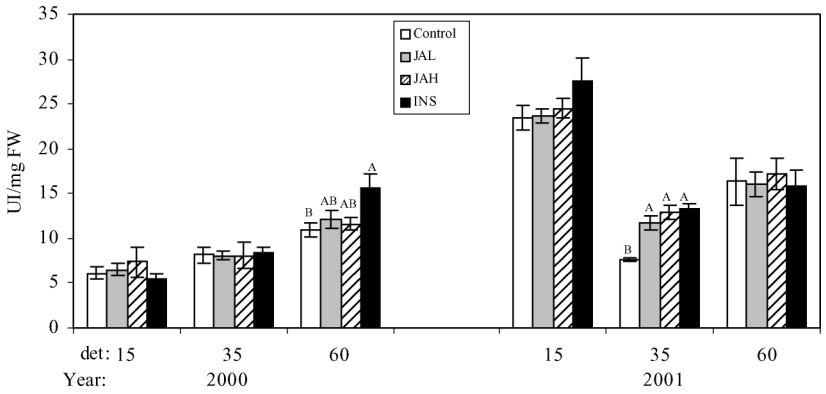
35
60
15
35
60
Year:
2000
2001

b)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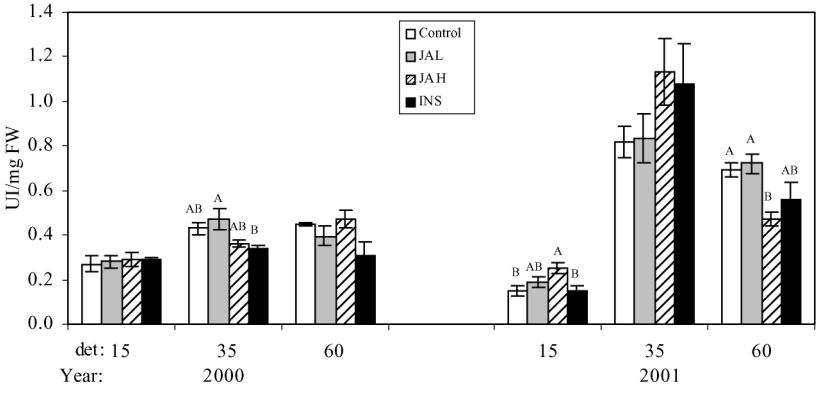
JAL
JAH
INS
UI/mg FW
det: 15
35
60
15
35
60
Year:
2000
2001

c)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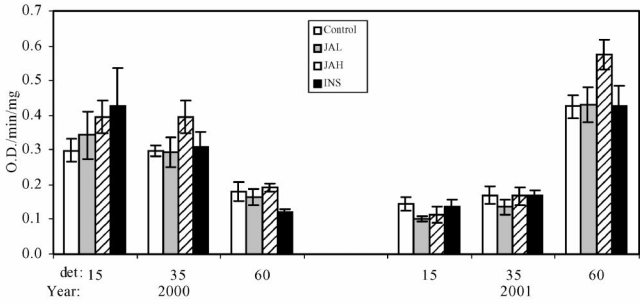
JAL
JAH
INS
UI/mg FW
det: 15
35
60
15
35
60
Year:
2000
2001

d)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JAL
JAH
INS
O.D./min/mg
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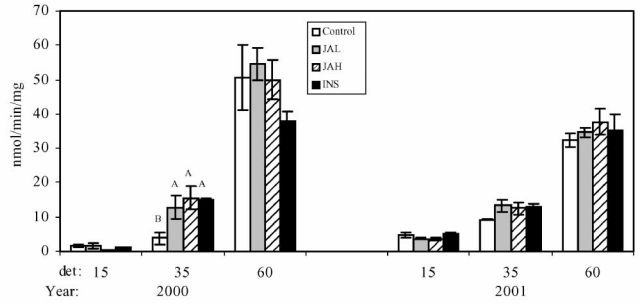
0.1
0.2
0.3
0.4
0.5
0.6
0.7
det: 15 35 60 15 35 60
Year: 2000 2001

e)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JAH
INS
nmol/min/mg
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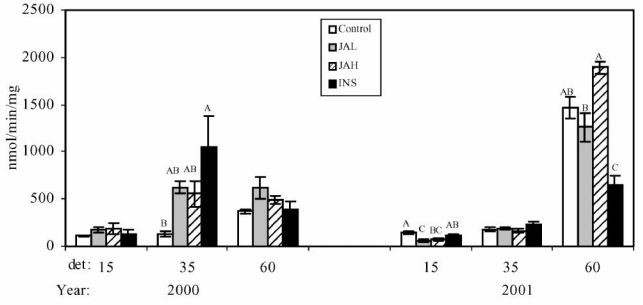
10
20
30
40
50
60
70
B
A
A
A
det: 15 35 60 15 35 60
Year: 2000 2001

f)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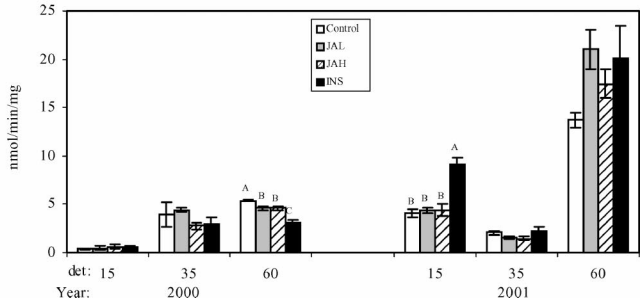
C
det: 15 35 60 15 35 60
Year: 2000 2001

g)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nmol/min/mg
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15
20
25
A
B
B
B
B
B
A
det: 15 35 60 15 35 60
Year: 2000 2001

In addition to treatment, effects of year on the seed head insect community were detected (Table 5). As mentioned, JA treatments had a negative effect on the abundance of all three components of the insect community, particularly PF insects. Drought probably reduced the attractiveness of JA-treated plants to insects by some undetermined mechanism. Previous studies indicate that drought (in tomato) or changes in irrigation scheduling (in cotton) affected defense-related plant chemistry and the performance or abundance of insect pests and predators on field grown plants (Flint et al., 1994, and references therein; English-Loeb et al., 1997). The results also suggest that any possible beneficial effect produced by a reduction in PF pests observed in JA-treated *A. hypochondriacus* plants in 2000 could have been counterbalanced by the negative effect that (low) JA treatments had on the PP insect community in seed heads. This effect on PP insects coincides, to some extent, with data that found no significant increase in the abundance of predaceous insects in JA-treated tomato plants (Thaler et al., 2001).

Aphids are a common insect pest of grain amaranths (Aragón-García and López-Olguín, 2001). In 2002, aphid numbers (*Macrosiphum euphorbiae* Thomas) in foliage of high JA-treated *A. hypochondriacus* plants were monitored throughout an 8-wk period. Aphid numbers were affected by T, S, and its interaction, and was consistent with the wide fluctuation in aphid numbers observed (Figure 1). Hence, high JA treatment reduced aphid numbers only in the youngest plants, whereas numbers in insecticide-treated plants were significantly higher on the last and next to last sampling dates (Table 3, Figure 1). The results from JA-treated plants were not in accordance, except on the first sampling date, with previous reports that showed that dosages of JA identical to those employed in this study decreased the preference, performance, and abundance of herbivores, including aphids, in tomato (Thaler, 1999a,b; Thaler et al., 2001). However, the fluctuating aphid numbers observed in JA-treated amaranth plants may reflect the lack of

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FIG. 4. Mean activities (±SE) of trypsin inhibitors (a), chymotrypsin inhibitors (b), α-amylase inhibitors (c), leucine aminopeptidases (d), polygalacturonases (e), β-1,3-glucanases (f), and chitinases (g), in leaves of untreated field grown *A. hypochondriacus* plants (control) or plants treated with a chemical insecticide (INS) or with low (0.5 mM, JAL) or high (1.5 mM, JAH) dosages of jasmonic acid (JA). Four leaves per plant were sampled from the upper third segment of five plants per experimental plot, 48 hr after each treatment. Treatments were applied on young plants (15 d after emergence and seedling thinning, "det"), developing plants (35 det), and mature plants (60 det). The results of two independent field experiments performed on consecutive years (2000 and 2001) are shown. Significant differences (at $P < 0.05$) between treatments within each developmental stage within each year are indicated by different letters above bars (ANOVA followed by Tukey test).

consistent responses to induced resistance usually shown by these insects. This behavior has been reported by other researchers in other plant species and appears to depend on the aphid species involved, its biotype within a species, and the plant development stage (Thaler, 1999b, and references therein). Conversely, the abrupt increase in aphid numbers observed in insecticide-treated plants on late sampling dates could be associated with a reduction in the natural abundance of predators (such as *O. insidiosus*; not shown) caused by insecticide treatments. This was inferred from the susceptibility to insecticide shown by this component of the insect community in seed heads of *A. hypochondriacus*, particularly in 2002 (Table 6, Figure 2c).

The application of exogenous JA did not have a negative impact on productivity measured as grain yield, seed germination percentage, and seed protein content (Figure 3a, e, and f). Seed vigor was not altered (results not shown). Even the low yields obtained from *A. hypochondriacus* plants treated with low JA in 2000 (807 kg/ha; Figure 3a) compare favorably with those reported by other workers (Aragón-García and López-Olguín, 2001), but are still lower than the 2–3 metric tons/ha reported for selected, high yielding, *A. hypochondriacus* lines in Mexico (Maldonado and Estrada, 1986). The production of reasonable yields, even under drought stress, was not surprising, considering that this is a defining characteristic of the *A. hypochondriacus* variety employed in this study (A. Borodanenko, personal communication). On the other hand, the higher yields produced by high-JA-treated plants in 2001 may reflect a higher level of tolerance to herbivory in plants not subjected to drought stress. Tolerance to herbivory is dependent, in addition to plant genotype, on environmental conditions, being favored when photosynthetic rates and water and nutrient uptakes are high (Agrawal, 2000). Thus, insect-derived damage could have affected yield, even in damage-tolerant *A. hypochondriacus* plants, under conditions in which they were unable to compensate adequately for herbivory losses due to drought stress.

The results shown in Tables 7 and 8 indicate that Y, DS, and its interaction were the factors that more significantly influenced chemical and plant productivity. The strong effect of Y suggests that the basal metabolism of *A. hypochondriacus* plants and, possibly, their response to JA (see below) are influenced by changes in environmental conditions. For instance, the higher levels of TIA detected in seed and leaves, in 2000, could be explained by the proposed protective role that has been assigned to the accumulation of trypsin inhibitors in a number of plant species undergoing desiccation (Reviron et al., 1992; Lopez et al., 1994; Welham et al., 1998; Lam et al., 1999). This possibility is supported by the observed accumulation of trypsin inhibitors in foliage of *A. hypochondriacus* plants subjected artificially to drought and salt stress (J. Délano-Frier and S. Valdés-Rodríguez, unpublished data). On the other hand, the physiological relevance of the changes in activity levels detected between years in other proteins, particularly in seed and foliar AAIA and CTIA levels, has yet to be determined. Lower germination efficiency in 2000 was probably also caused by drought stress, since seed dormancy is favored under

adverse environmental conditions (Bewley, 1997; Holdsworth et al., 1999). The difference in protein content in seeds is difficult to explain, but could have been caused similarly by the differential accumulation of stress-protective proteins (e.g., dehydrins and LEA proteins) (Xu et al., 1996). This possibility is under investigation.

The analysis of foliar chemistry showed that the levels of activity were influenced also by the development stage of the plant (Table 9, Figure 4). The relatively high levels of activity detected in some stages, particularly of protease and amylase inhibitors, coupled with the rather modest and sporadic increases in activity produced by exogenous JA, indicate that, in addition to their possible stress-protective role, these proteins might be part of a constitutive and developmentally regulated defense mechanism. In other plant species (e.g., potato, *Nicotiana tabacum* and *N. attenuata*), toxic proteins produced in induced defense responses are also known to accumulate as constitutive defenses (Gatehouse, 2002). Moreover, in plant systems subjected to heavy herbivore pressure, as appears to be the case of amaranth, inducible defenses are advantageous, and constitutive mechanisms are favored (Wolson and Murdock, 1990; Wittstock and Gershenzon, 2002).

Our results are in agreement with well-documented studies in other plant species that report that exposure to abiotic stress or changes occurring during development modify the activity and/or the JA-inducibility of several putative defense proteins (Vera et al., 1988; Cordero et al., 1994; Alarcon and Malone, 1995; Cipollini, 1997; English-Loeb et al., 1997; Cipollini and Redman, 1999; van Dam et al., 2001). Abiotic stress in plants can change plant chemistry and influence herbivore performance, both positively and negatively (Cipollini, 1997; English-Loeb et al., 1997); and increased resistance against insect herbivores can still be obtained in JA-treated plants even when simultaneous environmental stimuli reduce the activity of defense-related proteins (Cipollini and Redman, 1999).

It was difficult to identify in this study to what extent the changes detected in *A. hypochondriacus* plants were a consequence of exogenous JA. In general, the effects observed in JA-treated plants were rather modest and in agreement with the poor performance that chemical elicitors of defense responses in plants frequently have in field conditions (Lyon and Newton, 1999). Nevertheless, a number of findings from this study merit further research. First, JA did not have an adverse effect on productivity, and when the high dosage was applied in suitable growing conditions, it actually enhanced grain yield. Second, the application of JA had a significant effect on the insect community in foliage and seed heads. Third, drought stress had a powerful influence on all the variables. This implies that some of the chemical responses examined (e.g., TIA) could have a role in protection against adverse environmental conditions in addition to their suggested defensive role. The mechanism(s) responsible for the JA- and/or drought-induced changes remain to be determined. However, similar to the induction of jasmonate-induced proteins in barley (Lehman et al., 1995), osmotin in tobacco (Xu et al., 1994), and proteinase inhibitors in tomato (Dombrowski, 2003), a stress-induced activation of JA signalling and/or increase in endogenous JA levels, could be involved.

APPENDIX

METEOROLOGICAL CONDITIONS RECORDED IN THE EXPERIMENTAL FIELDS OF THE INSTITUTE OF AGRICULTURAL SCIENCES (UNIVERSITY OF GUANAJUATO)

Month	Temperature (°C)			Relative humidity (%)			Total rainfall (mm)	Mean evaporation (mm)	Mean isolation (h)
	Maximum	Mean	Minimum	Maximum	Mean	Minimum			
2000									
June	26.7	20.2	13.7	98.6	68.9	39.3	119.4	5.7	7.5
July	27.1	19.6	12.2	98.9	66.7	34.5	172.4	6.7	9.3
August	26.6	19.2	11.9	98.0	65.8	33.7	64.5	5.6	8.3
September	27.6	19.1	10.6	96.7	62.9	29.2	40.7	5.7	9.0
October	27.3	18.2	9.1	96.4	61.7	27.1	15.4	4.8	8.5
November	26.2	16.7	7.2	95.4	60.7	26.1	0.0	4.9	8.8
December	23.6	14.1	4.3	93.2	60.5	27.7	9.2	3.5	8.1
2001									
June	27.5	20.5	13.5	95.9	64.7	33.6	205.0	6.60	8.7
July	26.5	19.6	12.8	97.5	65.2	33.0	217.8	6.40	9.2
August	26.6	19.5	12.5	97.1	65.1	33.1	260.9	5.96	8.2
September	26.4	19.2	12.0	97.1	64.3	31.5	128.7	5.10	8.0
October	26.0	16.9	7.9	94.8	62.0	29.2	36.0	4.50	9.4
November	25.2	14.9	4.6	96.9	58.9	20.9	3.5	4.00	9.6
December	24.0	14.6	5.2	94.7	61.0	27.4	1.4	3.39	7.5

APPENDIX CONTINUED

Month	Temperature (°C)			Relative humidity (%)			Total rainfall (mm)	Mean evaporation (mm)	Mean isolation (h)
	Maximum	Mean	Minimum	Maximum	Mean	Minimum			
2002									
June	30.0	21.8	13.6	93.4	61.7	30.0	71.8	7.37	8.8
July	26.2	19.6	13.0	98.0	67.5	36.9	237.8	6.41	6.9
August	26.6	19.4	12.3	97.1	64.7	32.3	201.0	7.08	9.3
September	26.0	19.6	13.3	97.4	67.6	37.8	129.6	4.82	6.5
October	27.3	19.1	10.9	97.7	63.9	30.1	53.4	4.25	8.0
November	24.4	15.3	6.2	96.1	58.2	20.6	50.9	3.46	7.1
December	23.9	13.8	3.8	96.2	59.6	23.0	0.3	3.00	7.5
1979–2002									
June	28.4	21.3	14.3	84.7	58.4	32.0	117.4	7.79	8.4
July	26.1	20.0	13.7	90.0	63.2	36.6	159.8	6.43	7.7
August	25.9	19.5	13.2	89.6	63.3	36.3	168.5	6.41	8.0
September	25.5	18.9	12.4	90.5	62.5	34.8	107.4	5.62	7.4
October	25.5	17.5	9.5	86.3	57.1	29.3	43.4	5.10	8.2
November	24.8	15.7	6.6	86.1	55.7	26.1	10.1	4.40	8.5
December	23.2	14.3	5.3	84.3	55.4	26.5	8.8	3.80	7.9

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GENETIC ANALYSIS OF BENZOQUINONE PRODUCTION IN *Tribolium confusum*

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Abstract—Many species of tenebrionid beetles produce and secrete benzoquinones from specialized prothoracic and postabdominal glands. *Tribolium confusum* produces two compounds methyl-1,4-benzoquinone (MBQ) and ethyl-1,4-benzoquinone (EBQ). These compounds are hypothesized to function as external defense compounds, killing microbes and deterring predators, and their ability to evolve by natural selection depends on both selection and the genetic vs. environmental contribution to phenotypic variation. We crossed a strain of *T. confusum* that produces high quantities of benzoquinones, b-Pakistan, with a low-producing strain, b-+, and measured both the internal and external quantities of MBQ and EBQ for the two extreme strains and their F_1 progeny. Internal amounts show a clear pattern of inheritance, with at least 50% of the phenotypic variation attributed to genotype. Additive and dominance coefficients for internal amounts indicate that the trait is additive with no significant dominance. In contrast, external quantities show little pattern of inheritance. The role of genetics and environment in determining quantities of secretory defensive compounds is important to elucidating the ecology and evolutionary potential of chemical defenses.

Key Words—Defense compounds, genetic vs. environmental components, *Tribolium*, benzoquinones, evolutionary adaptation.

INTRODUCTION

Many organisms produce constitutive defense chemicals. The evolution of constitutive defensive compounds is likely to be affected by the costs and benefits

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associated with producing them. Species from all taxa metabolize and excrete specific chemicals that help defend them from other organisms, yet there is a lack of information on the genetics of animal defensive secretions (Berenbaum, 1995). Studies on defensive secondary metabolites seem to be limited mostly to plants (Frey et al., 1997; Vershinin, 1999; Agrawal et al., 2002; Kliebenstein et al., 2002). However, there is some information on the secretions of other beetles. A genetic study of within population variation found a considerable part of the variation in chemical defense of the leaf beetle, *Oreina gloriosa*, is genetically determined (Eggenberger and Rowell-Rahier, 1991, 1992, 1993). Adult *O. gloriosa* have at least 16 secretion components; about half of these have significant genetic components to their variation (Eggenberger and Rowell-Rahier, 1991, 1992, 1993). The estimated heritabilities are about half of those that have been done for two beetle species, and the two-spot ladybird, *Adalia bipunctata* (Holloway et al., 1993). In general, studies on the secretions of other insects have concentrated on identification, quantification, and possible function rather than genetics (Eisner and Meinwald, 1966; Blum, 1981). Since their presence was first noted in the 1800s, these are the types of studies that have been done on the benzoquinone compounds found in *Tribolium* beetles.

Benzoquinone derivatives are produced and secreted by hundreds of species of invertebrates including arachnids, diplopods, and insects (Blum, 1981). Flour beetle species in the genus *Tribolium* excrete up to three different benzoquinones: methyl-1,4-benzoquinone (MBQ), ethyl-1,4-benzoquinone (EBQ), and methoxybenzoquinone (Blum, 1981). *Tribolium confusum* produces both MBQ and EBQ, but not methoxybenzoquinone. Complex and specialized secretory organs are used for the production and storage of these toxic chemicals (Roth and Stay, 1958; Happ, 1968). The quinones are produced in paired prothoracic and postabdominal glands and are discharged when the beetles are irritated. Benzoquinones are effective at deterring potential predators such as rats, toads, and cockroaches, although they do not guarantee the predator still will not eat them (Roth, 1941; Roth and Stay, 1958). The compounds are also effective at killing the beetles themselves when an absorbing substrate such as flour media is not present (personal observation). Observations that a low-producing "mutant" strain that had only one-twentieth of normal levels had difficulty with fungi in its flour habitat indicated a possible antimicrobial function (Engelhardt et al., 1965). Also, large amounts of both MBQ and its hydroquinone form are effective against Gram-positive bacteria (Geiger, 1946). Although the traits are taxonomically widespread and relatively complex, little has been done on the genetics of secretory defense compounds (Blum, 1981), and the function of these compounds is still unclear. However, because of autotoxicity there is a cost to the production of the compounds (Roth and Howland, 1941, personal observation).

Genetically distinct strains of *T. confusum* demonstrate significant differences in the internal and external quantities of both MBQ and EBQ. The b-Pakistan strain

produces nearly four times the quantity of total benzoquinones as the b-+ strain (Yezerski et al., 2000). These two strains represent the extremes from four strains (b-+, b-I, b-IV, and b-Pakistan) measured in a previous study. Quantitative traits such as these are usually influenced by both genetic and environmental factors, therefore, we tested for a genetic basis to internal and external quantities and estimated the additive and dominance components of the traits by examining the two genetically distinct strains and their F_1 progeny.

METHODS AND MATERIALS

Beetles. Parental beetles were obtained from inbred *T. confusum* stocks of the strains b-Pakistan (b-Pak) and b-+ (see Stevens, 1989, for the origin of b-+; b-Pak was obtained from Dr. Ralph Howard, Kansas State University). These parental strains were inbred for six generations by full-sibling mating from the laboratory stocks before we began our experiment to ensure a genetic consistency among members of the same strain.

Crosses. Six single pair crosses of b-+ $\times$ b-Pak (female $\times$ male) and eight reciprocal single pair crosses were set up in 1 dram glass vials containing 1 g of fine sifted whole wheat flour with 5% by weight Brewer's yeast. Adult beetles, about 1 mo post eclosion, were allowed to mate and lay eggs for 3 d, then the parents were assayed for their internal and external benzoquinone quantities. The progeny, raised in darkened incubators at 70% RH and 29°C, were measured approximately 1 mo after eclosion. Three male and three female progeny from each cross were measured. All beetles were randomly chosen for measurement to negate possible effects of circadian controls.

Measurement of Benzoquinones. Internal and external MBQ and EBQ quantities of individual adult beetles were assayed using high performance liquid chromatography (HPLC) (Pappas and Morrison, 1995; Yezerski et al., 2000). Authentic standards were used to calibrate the instrument. MBQ is commercially available (Aldrich Cat. #21,131-1). EBQ was synthesized by modifying a method for adding side groups to benzoquinones (Yamada and Hosaka, 1977).

EBQ degrades at a rate of 3% per min and MBQ degrades at a slower rate of 2.5% per min under indoor light conditions and 22°C (Yezerski et al., 2000). To minimize degradation, compounds were quickly ($<$5 min) extracted and immediately injected into the HPLC. External benzoquinone quantities were assayed from individual beetles rinsed with 1 ml HPLC grade methanol in an Eppendorf tube by shaking for 8 min. The beetle was transferred into a second Eppendorf tube containing 1 ml of fresh HPLC grade methanol, homogenized, shaken for 1 min, and filtered by centrifugation for 2 min. The supernatant represented the internal benzoquinone quantity.

Samples were injected through a 50 μl loop into a 20 $\times$ 4.6 mm Spherisorb S10 ODS1 column that used a 1:1 methanol:water mobile phase at a flow rate of

1.7 ml/min. Using a Waters 994 detector, peaks were identified at a wavelength of 246 nm. The relationship between concentration and peak height was determined with MBQ and EBQ standards in concentrations bracketing the previously measured extremes from the beetles. Standards were run every 40 injections to insure consistency. The average r^2 value between concentration and measured peak height over all standards and all assays was 0.9993 for MBQ and 0.9996 for EBQ. These values demonstrate that the curves accurately reflected benzoquinone concentrations as measured in milligrams per beetle.

Data Analysis. To achieve a normal distribution of the data, arcsine transformation was necessary for all internal quantities. Natural log transformation was used to normalize the external quantity distributions. For each trait, the family mean was used in the analysis. The analysis determined whether the benzoquinone values showed a genetic pattern of inheritance. They also estimated the additive and dominance components of the genetic variation, when appropriate, in a manner similar to Stevens (1989; see also Stevens, 1994). Genetic effect is a result of a regression of gene dose (b-+ = 0, progeny = 1, b-Pak = 2) on corrected values for the benzoquinones. This method uses a joint scaling test to estimate additive and dominance effects (Mather and Jinks, 1977). Additive values are derived from the slope of the aforementioned regression. Dominance deviations were determined by comparing actual values of the progeny to the predicted mid-parent values using a chi-square test.

RESULTS

Internal Benzoquinones. Internal quantities of both MBQ and EBQ show a genetic component and pattern of inheritance. Values for the progeny are intermediate to the parents (Figure 1), and at least half of the variance in internal quinone amounts is explained by gene dose (Table 1, 51% for MBQ and 59% for EBQ). Gene dose is the genetic background of the trait as defined by allelic state (Falconer and Mackay, 1996). Internal MBQ and EBQ have statistically significant additive effects, but the dominance coefficients are not significant (Table 1).

External Benzoquinones. External benzoquinone amounts are only about 7.5% of internal amounts. Genetics has a small, but statistically significant, effect on external quantities of EBQ, but not MBQ (Table 1). The cross type is not significant for MBQ, but is for EBQ. External quinone amounts show little correlation with gene dose (Figure 1 and Table 1, $r^2 = 0.20$ for external EBQ and 0.12 for external MBQ). Variances for external benzoquinones are greater and more variable than for internal benzoquinones.

Internal vs. External Benzoquinones. Scatterplots of the correlations between internal and external values for each benzoquinone grouped by family are shown in Figure 2. These plots show no relationship between internal and external amounts of the compounds.

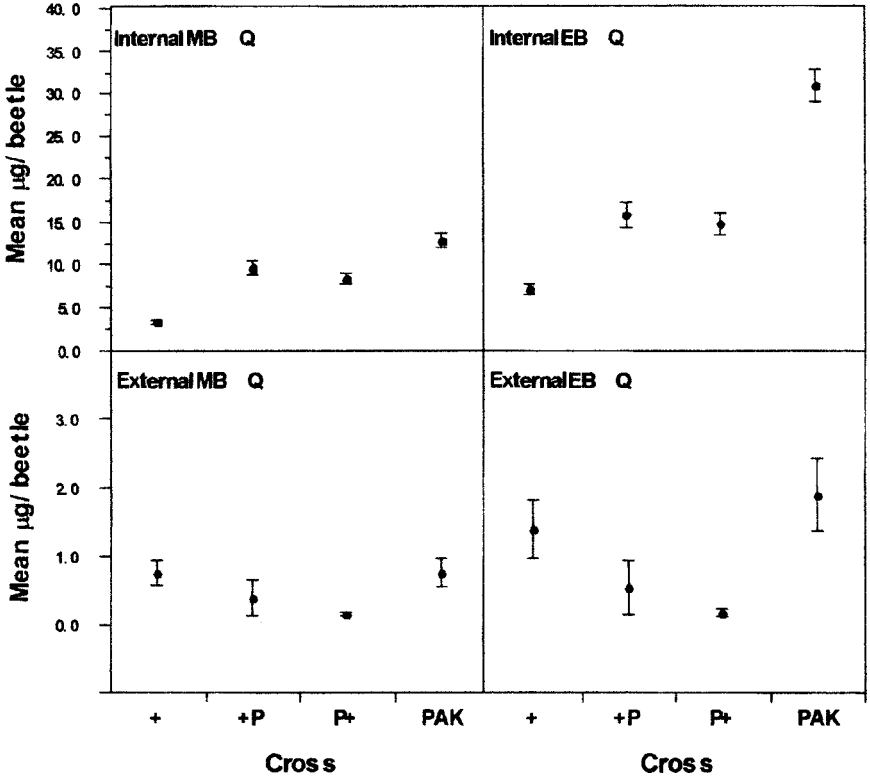

FIG. 1. External and internal quantities (±SE) of two secretory defense compounds, methyl-1,4-benzoquinone (MBQ) and ethyl-1,4-benzoquinone (EBQ) shown by source (+: from b-+ parents ($N = 28$); +P: from progeny of a cross between a b-+ female and a b-Pakistan male ($N = 48$); P+: progeny from the reciprocal cross ($N = 36$); and PAK = from b-Pakistan parents ($N = 28$)).

DISCUSSION

Partitioning phenotypic variation into genetic and environmental components, and documenting the pattern of inheritance of secretory defense compounds helps to elucidate the potential of such traits to respond to natural selection. Internal quantities of benzoquinones demonstrate an additive pattern of genetic inheritance with incomplete dominance. Progeny means are intermediate between parental means. In contrast, external quantities appear to be governed by environmental effects. Previous work indicated that genetically distinct strains of *T. confusum* have significant differences in internal amounts, but generally do not show the same pattern for their external amounts (Yezerski et al., 2000).

TABLE 1. ADDITIVE AND DOMINANCE COEFFICIENTS FOR THE INHERITANCE OF BENZOQUINONES

	Estimate of genetic effect		Additive component		Dominance deviation	
Measure	r^2	P-value	Mid-parent	Additive effect	χ^2	P-value
Internal MBQ	0.51	<0.001	0.29	0.10	0.002	>0.05
Internal EBQ	0.59	<0.001	0.45	0.17	0.014	>0.05
External MBQ	0.12	0.08	3.17	−0.45	0.31	>0.05
External EBQ	0.20	0.009	3.64	−0.25		

We examined naturally occurring variation among populations. We did not select for high and low levels, but rather used strains that naturally differ in benzoquinone production and inbred them to lessen genetic variation among individuals of the same strain. Previous studies of variation in quinone quantities examined mutant stains that showed greatly reduced benzoquinone quantities (Engelhardt et al., 1965). The genetic component of variation is relatively large for a phenotypic trait. However, like most quantitative traits, quinone quantities are influenced by both genetics and environment (ex. for insects; see Fanara et al., 2002; Gadau et al., 2002; Gleason et al., 2002; Gockel et al., 2002; Lapidge et al., 2002; Tahoe et al., 2002). Interestingly, high quantities of benzoquinones are rare in these beetles. Indeed, statistical transformation was required to improve the fit to a normal distribution since the distribution was so leptokurtic. High quantities might be selected against because they are costly to produce and maintain, and/or deleterious to the beetles themselves.

Costs of producing quinones include the raw materials, including glucosidase, as well as enzymes, namely phenoloxidase. These molecules are used in immune defenses in addition to quinone production, tanning of the cuticle, and in digestion (Happ, 1968; Singer et al., 1975; Sugumaran and Semensi, 1987; Sugumaran et al., 1992). Therefore, trade-offs to other systems in order to produce quantities of benzoquinones might be necessary. Current studies in our laboratory are investigating the possible trade-offs between chemical production and cuticle color, as well as the effects on parasite susceptibility that has been noted previously for *T. castaneum* (Yan and Phillips, 1996).

Although the costs of quinone production are relatively unknown, several costs of maintaining quinones have been documented. Quinones are harmful to a wide range of organisms, including beetles themselves. We consistently observe that when a few individuals from the high production b-Pak strain are maintained in an otherwise empty vial, they all die within 24 hr, whereas beetles from other lower-producing strains show almost 100% survival. High levels have also been reported to interfere with successful development (Roth and Howland, 1941; Sokoloff, 1977). Negative effects on host physiology might be balanced

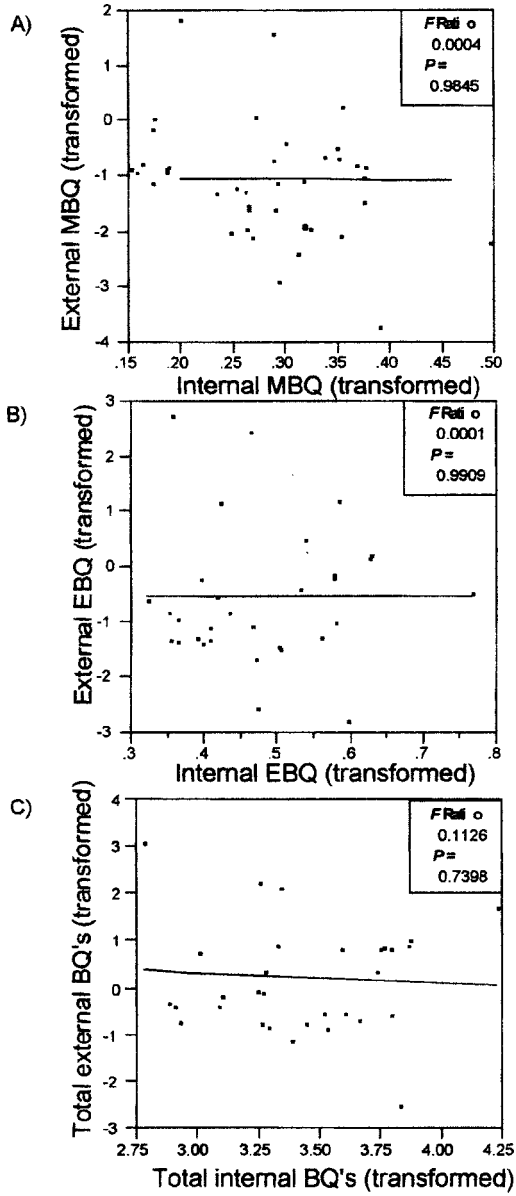

FIG. 2. Scatterplots showing the relationships between internal and external benzoquinones. Shown is (A) internal vs. external MBQ, (B) internal vs. external EBQ, and (C) internal vs. external total benzoquinones. Points represent family means transformed as stated in the text.

by selection for the positive effects, including predator deterrence or reduction of microbes. Although rats and toads that either eat the beetles or biscuits made from flour that had contained beetles can develop cancer, the effectiveness of the compounds to actually deter such predation has not been demonstrated (El-Mofty et al., 1988, 1992). We are currently investigating the effects of the compounds on rat feeding behavior. A deleterious indirect effect on the microbial environment has also been suggested (Geiger, 1946; Engelhardt et al., 1965). We are compiling data that shows that these compounds may be effective against bacterial species commonly found in flour. In general, the possible functions of the compounds have yet to be explored, and the purported trade-offs are still being investigated.

External quantities did not have the strong genetic basis that the internal amounts had. Metabolism, internal catabolism, and secretion determine internal concentrations. External amounts are partially determined by internal amounts, but also the depletion of surface amounts from volatilization of the compounds, absorbency of the chemicals into the surrounding flour media, and degradation of the benzoquinones into their constituent hydroquinones. Since the beetles are rinsed at one point in time, our external amounts represent only those benzoquinones secreted and still present on the exoskeleton during rinsing. It may be that beetles store quinones and secrete them when necessary, or that the strains differ in the excreted amounts, and that external quantities on the surface of the beetle are not related to the excreted amount. This is further supported by scatterplots that show no correlation between internal and external amounts within family (Figure 2). In other words, the glands may only be able to secrete a given amount at any time regardless of genetic strain, but internal production may allow more secretion over time, thus increasing the quantity in the surrounding environment. The larger differences in variance for external amounts also suggests an environmental component that is stronger than a genetic component. Beetles poked with a paintbrush will exude more chemical when disturbed (Roth, 1941). However, other environmental components may also play a part such as light cycles, temperature, and other forms of disturbance. Of note is the fact that the parental beetles were measured 1 mo before the progeny in order to ensure similar ages during measurement. Differences in the environment of the lab between these two months may account for the generally lower external values of the progeny vs. the parents. Other methods that measure secretions over time rather than the single time point method used here may better clarify the role of environment (Yan and Phillips, 1996).

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THE CHEMISTRY OF POLLINATION IN SELECTED BRAZILIAN MAXILLARIINAE ORCHIDS: FLORAL REWARDS AND FRAGRANCE

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Abstract—We report the chemical composition of the floral rewards and the fragrance of 10 *Maxillariinae* (Orchidaceae) species. The species that offer rewards (labellar secretions) are usually scentless, the rewards being collected by bees. Chemical analyses revealed that the major chemical class of compounds present in the labellar secretions are triterpenoids. The rewardless *Maxillariinae* flowers were usually scented, and chemical analyses of their volatiles revealed that they were composed of mono and sesquiterpenoids.

Key Words—Orchids, Maxillariinae, pollination, floral rewards, labellar secretion, fragrance, gas chromatography–mass spectrometry, NMR analysis, terpenoids, 3β-hydroxy-cycloart-24-en-26-al.

INTRODUCTION

A significant proportion of the angiosperms is pollinated by animal pollen-vectors (Endress, 1994). These pollinators are attracted by the flower display (a combination of flower shape, color, and fragrance), and also by the so-called "flower rewards" (Endress, 1994). The term "reward" is herein used to denote those flower parts or secretions that can somehow be eaten, foraged, or stored by pollen-vectors for use in their life cycles. These flower products enhance flower visitation by pollinators and, during foraging activities, pollination takes place. During successive

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flower visitations, the pollen first adheres to the body surface of the pollen-vector and is subsequently transferred to conspecific stigmatic surfaces, thus promoting pollination.

Orchidaceae are remarkable among the angiosperms because of their specialized flower morphologies and life cycles (Kullenberg, 1961; Dressler, 1993, and references therein). The more basal orchids (subfamily Apostasioideae) offer pollen as a reward to their pollinators (Kocyan and Endress, 2001). In more advanced Orchidaceous flowers, the pollen is generally packed in hard, discrete pollinia, thus hindering its use as a flower reward (Dressler, 1993). Yet, a significant number of orchid flowers offer no reward to the pollinators (Van der Pijl and Dodson, 1966; Dressler, 1993). The life cycles of these species involve pollination processes (often bizarre) where flowers deceive the pollinators into searching for food or sexual partners (provoking the so-called "pseudocopulation") (Van der Pijl and Dodson, 1966; Dressler, 1993). Among reward-offering orchid flowers, disparate kinds of flower rewards, such as nectar, oils, waxes, starchy trichomes (often called "pseudopollen" because of the pollen-like appearance), and aromatic oils can be found (Van der Pijl and Dodson, 1966; Dressler, 1993; Reis et al., 2000).

The neotropical subtribe Maxillariinae (with ca. 800 spp., according to Dressler, 1993) poses special problems to taxonomists, due to its size and diversity. Whereas the subtribe as a whole is monophyletic (Whitten et al., 2000; Holtzmeier et al., 1998), the genus *Maxillaria* Ruiz et Pavón (ca. 600 spp. according to Dressler, 1993) is largely unnatural, with minor (and currently accepted) genera such as *Trigonidium* Lindl., *Mormolyca* Fenzl., *Chrysocycnis* Linden et Reichb. f., and *Cryptocentrum* Benth. et Hook nested within. The Brazilian species of *Maxillaria* were grouped by Pabst and Dungs (1977) into several "alliances." As currently accepted, the genus *Maxillaria* includes both reward-offering and rewardless species (Dressler, 1993; Singer and Cocucci, 1999; Singer, 2002). Some species, such as *M. parviflora* (Poepp. et Endl.) Garay and *M. coccinea* Lindl., offer nectar droplets on the surface of their lips (Singer, 2002). Several Ecuadorian *Maxillaria* species of the "Grandiflora" alliance produce pads of detachable, moniliform hairs that, owing to their pollen-like farinaceous appearance, have been called "pseudopollen" (Davies, 1998; Davies et al., 2000). Preliminary reports on waxy secretions by flowers of *Maxillaria cerifera* Barb. Rodr. and *M. brasiliensis* Brieger et Bicalho have been recently published (Singer, 2002), and at least the latter species is pollinated by Meliponini bees that gather the labellar secretions on their corbiculae (Singer, 2002). The labellar secretions of *M. cerifera*, *M. acuminata* Lindl., and *M. notylioglossa* Rchb. f. were identified as lipoidal through histochemical analysis (Davies et al., 2003). However, to date, no details of the chemical composition of these flower rewards are available.

Many *Maxillaria* species, such as *M. picta* Hook., offer no reward to their pollinators (Singer and Cocucci, 1999). The strongly honey-scented flowers of

M. picta are pollinated by Meliponini bees that are apparently capable of learning that the flowers are rewardless (Singer and Cocucci, 1999). Consequently, the pollination process only takes place during the first days of anthesis (Singer and Cocucci, 1999; Singer, 2002). The rewardless flowers of *Trigonidium obtusum* Lindl. are pollinated by sexually excited drones of *Plebeia droryana* (Meliponini), which attempt copulation with the sepals or the tip of the lateral petals (Singer, 2002). A multidisciplinary task-group involving scientists from different universities and countries (www.flmnh.ufl.edu/natsci/herbarium/max/) is actively working on the resolution of the phylogeny of the subtribe Maxillariinae as a whole. In this context, our team at the Universidade Estadual de Campinas (Unicamp) is working with molecular biology, flower, and vegetative morphology, anatomy, chemical analyses of fragrance, and flower rewards of Brazilian Maxillariinae. Since *Maxillaria* as currently accepted is a grossly unnatural genus, massive generic rearrangements are to be expected in the near future. In this context, there is an urgent need to obtain sets of characters from any source that would help in the diagnosis of the clades obtained through molecular data. The present contribution is the first report on the nature and chemical composition of Maxillariinae floral rewards (labellar secretions) and fragrances, and its primary aims are (1) to characterize and report on the chemical compositions of the flower rewards of some selected Brazilian *Maxillaria* species (*Maxilliaria cerifera*, *Maxillaria brasiliensis*, and *Maxillaria friedrichsthalii* Rchb. f.) and (2) to investigate the floral fragrances of seven additional Maxillariinae (*Maxillaria rufescens* Lindl., *Maxillaria marginata* Fenzl., *Maxillaria gracilis* Lodd., *Trigonidium* cf. *turbinatum*(Rchb. f.), *Maxillaria picta* Hook., *M. jenischiana* (Rchb. f.) C. Schweinf., and *Trigonidium obtusum* Lindl.). Among these seven additional species, only *M. rufescens* produce some reward (trichomes) to its pollinators. The reward-lacking *Maxillaria* species included in this study display remarkable fragrance emissions, which apparently elicit behavioral responses in food-seeking hymenoptera (Singer and Cocucci, 1999; Singer and Koehler, personal observation).

METHODS AND MATERIAL

Plant Material. Fresh flowers were obtained from plants cultivated at the Universidade Estadual de Campinas (Unicamp), the ESALQ Orchidarium, and the Orchidarium of the Instituto de Botânica de São Paulo (*M. marginata* [IBt 247], *Maxillaria cerifera* [Koehler 219], [Koehler 220], and [Koehler 223], *M. gracilis* [IBt 5116], *Maxillaria rufescens* [Koehler 204], *Trigonidium obtusum* Lindl. [Singer s.n.], *Maxillaria picta* [Singer s.n.], *Trigonidium* cf. *turbinatum* [ESA 8239] and *Maxillaria brasiliensis* [Koehler 170]). Plant vouchers were deposited at UEC, ESA, and SP Herbaria.

Extraction. The labella of 46 *Maxillaria cerifera* flowers, 5 *Maxillaria brasiliensis*, and 2 *Maxillaria friedrichsthalii* were extracted with diethyl acetate. The

organic solvent was then evaporated at reduced pressure. Derivatives of the crude extract (1 mg) were prepared using N,O-bis(trimethylsilyl)-trifluoroacetamide (40 μl) and pyridine (500 μl) for 1 hr at room temperature.

GC/MS Analysis. GC analysis was conducted using an Hewlett-Packard 6890 apparatus fitted with an HP-5 fused silica capillary column (30 m, 0.25 mm, 0.25 μm.) A sample volume of 0.5–1 μl was injected, and pressure programming was used to maintain a constant flow (1 ml/min) of the helium carrier gas. The mass spectrometer Hewlett-Packard 5973 was used in the EI mode (ionization energy of 70 eV) and set to scan the mass range of 50–700 amu at the rate of 2.94 scans per sec. The interface temperature was maintained at 300°C. The resulting data were processed by the Hewlett-Packard Chemstation Software package. Temperature programming for the reward analysis was from 50°C (5 min) to 310°C at 60°C/min plus 20 min at the final temperature. The injector temperature was 260°C. Fragrance analyses were performed with temperature programming from 50° to 290°C at 4°C/min with injector temperature of 240°C.

Isolation. The extract (35 mg) was purified by silica gel column chromatography (10 g), eluted with hexane and hexane containing increasing amounts of ethyl acetate. Similar fractions obtained by TLC were combined and the solvent evaporated, producing four fractions ($a = 2$ mg, $b = 2$ mg, $c = 5$ mg, $d = 18$ mg).

NMR Analyses. ^{1}H NMR spectra were recorded on a Varian INOVA 500 (499.88 MHz) spectrometer, $CDCl_3$ was used as the solvent, with Me_4Si (TMS) as internal standard. ^{13}C-NMR analyses were recorded on a Varian INOVA 500 (125.69 MHz) spectrometer. $CDCl_3$ (77.0 ppm) was used as internal standard. Methyl, methylene, methine, and carbon nonbonded to hydrogen were discriminated using DEPT-135° and DEPT-90° spectra (Distortionless Enhancement by Polarization Transfer). 2D NMR spectroscopy was performed with standard H,H correlation and H,C correlation pulse sequences (HSQC for the one bond correlation and HMBC for the long range correlations) available in the spectrometer.

Fraction c: *Cycloartenal*. $[\alpha]_D^{23} = +23.5°$ (*c*, 3,6 in CHCl3). IR ν_{max}^{KBr} cm^{-1}: 3408 (OH); 2855, 1683 (C=O). EIMS (70 eV): *m/z* (%) = 440 (M^+, 6%), 422 ($M-H_2O$, 31%), 407 (53%), 379 (35%), 353 (18%), 300 (29%), 55 (100%). ^{1}H NMR (499.88 MHz, $CDCl_3$): δ 1.58 and 1.26 (H-1), 1.58 and 1.78 (H-2), 3.28 (*dd*, H-3 α, $J_{H7\alpha} = 12$ Hz, $J_{H7\beta} = 4.0$ Hz), 1.30 (H-5), 0.80 and 1.60 (H-6), 1.08 and 1.32 (H-6), 1.52 (H-8 β, $J_{7\alpha} = 12$ Hz, $J_{H7\beta} = 4.5$ Hz), 1.10 and 1.98 (H-11), 1.63 (H-12), 1.43 (H-15), 1.32 and 1.94 (H-16), 1.61 (H-17), 0.97 (*s*, H-18), 0.34 (*d*, H-19 *exo*, $J = 4.2$ Hz), 0.55 (*d*, H-19 endo, $J = 4.2$ Hz), 1.45 (*d*, H-20), 0.93 (H-21), 1.24 and 1.62 (H-22), 2.0 and 2.3 (H-23), 1.24 and 1.62 (H-24), 6.48 (*dt*, H-24, $J = 7$ Hz, $J = 1$ Hz.), 9.39 (*s*, H-26), 1.76 (*s*, H-27), 0.90 (*s*, H-28), 0.97 (*s*, H-29), 0.81 (*s*, H-30). ^{13}C NMR (125.69 MHz, $CDCl_3$): δ 31.96 (C-1), 30.48 (C-2), 78.82 (C-3), 40.48 (C-4), 47.08 (C-5), 21.10 (C-6), 26.01 (C-7), 47.98 (C-8), 19.94 (C-9), 26.06 (C-10), 26.06 (C-11), 32.88 (C-12), 45.37 (C-13), 48.83 (C-14), 35.52 (C-15), 28.20 (C-16), 52.18

(C-17), 18.08 (C-18), 29.71 (C-19), 35.94 (C-20), 18.10 (C-21), 34.77 (C-22), 30.36 (C-23), 155.65 (C-24), 139.08 (C-25), 195.46 (C-26), 9.17 (C-27), 25.44 (C-29), 14.00 (C-30).

Headspace. Analysis of the floral volatiles was performed by trapping them from 11:00 in the morning to 15:00 in the afternoon (this time schedule was adopted in agreement with the observed pollinator visitation periods) using dynamic headspace methodology (Kaiser, 1993). A 7-cm long glass vessel with an internal diameter of 4 cm was constructed to contain the orchid flowers, with an opening to insert the flower and an outlet on the opposite side connected to an absorption trap containing Porapak Q 80–100 mesh (50 mg) and glass wool at both ends, previously treated with solvent and activated at 150°C under vacuum. The scented air was drawn through the trap by means of a battery-operated pump (500 ml/min). The trapped components were eluted from the matrix with dichloromethane (1 ml) and concentrated in a N_2 current (0.2 ml), 0.5 μl of this solutions being injected into the GC/MS. Identification of fragrance compounds was made on the basis of a comparison of their retention indices (Van der Dool and Kratz, 1963) as well as by computer matching of the mass spectra obtained with those in the Wiley 275 mass spectra library of the GC/MS data system and other published mass spectra (Adams, 1995).

RESULTS AND DISCUSSION

Chemistry of Maxillaria Rewards. Purification of *M. cerifera* labellar reward produced 5 mg of a crystalline compound with a molecular ion at m/z 440 and 30 carbon signals in the ^{13}C NMR spectrum, characteristic of triterpenes. The ^{1}H NMR spectrum showed two doublets at 0.55 and 0.34 ppm corresponding to one hydrogen, both being characteristic of cyclopropane hydrogens, and a carbinolic hydrogen at 3.28 ppm, characteristic of a β-hydroxyl at position 3. The hydrogen at 6.48 ppm was connected to the carbon resonating at 155.65 ppm and scalarly coupled to hydrogens at 1.58 and 1.76 ppm (both being connected to a carbon at 30.36 ppm) and to a deshielded hydrogen at 9.39 ppm (connected to an aldehyde carbon at 195.46 ppm). These data suggest the structure of a triterpene with an α,β unsaturated aldehyde and a cyclopropane ring. A search of the literature showed several triterpenes possessing cyclopropanes, but only 3β-hydroxy-cycloart-24-en-26-al (Anjaneyulu and Prasad, 1985) possessed an α,β-unsaturated moiety. Unfortunately, this compound was never fully assigned, so we are now reporting the complete carbon and hydrogen assignment based on 1D (^{1}H, ^{13}C, DEPT 135 and 90) and 2D NMR (H,H COSY, H,C HSQC, H,C-HMBC) (Figure 1). The *E* stereochemistry of the double bond at carbon 24–25 was based on the 4% enhancement of the aldehyde hydrogen signal by excitation of the H-24 in the nOe experiment (NOESY 1D) and vice versa. The *cis* fusion of the B/C rings was confirmed by nOe experiments in which irradiation of the cyclopropane hydrogen

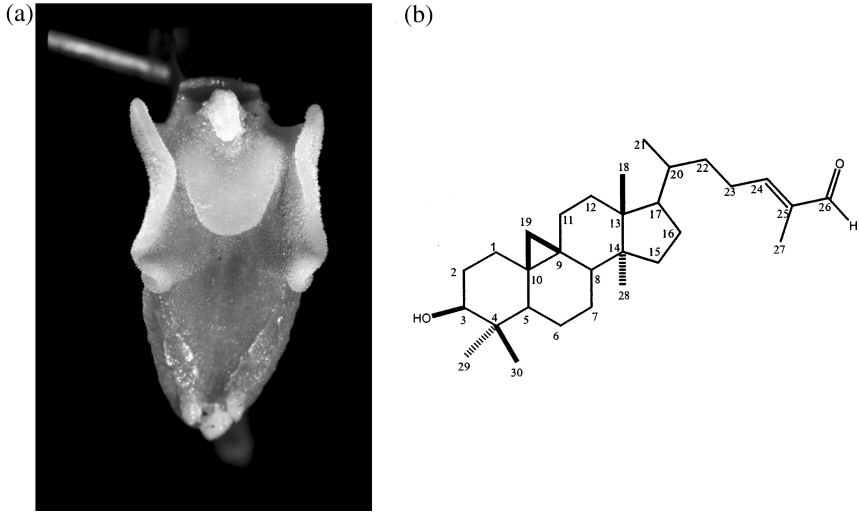

FIG. 1. (a) *Maxillaria cerifera* labellum containing crystals of the triterpenoid mixture; (b) structure of 3β-hydroxy-cycloart-24-en-26-al.

at 0.55 ppm produced an enhancement of the H-8 (3%, 1.52 ppm), H-18 (2%, 0.97 ppm), H-30 (5%, 0.81 ppm), and H-19 (10%, 0.34 ppm) signals.

Identification of the three remaining triterpenes (2 mg of a mixture), also possessing cyclopropane rings (two shielded hydrogens at 0.35 and 0.57 ppm as visualized in the ^{1}H NMR of the mixture), was investigated by GC/MS as cycloartenol derivatives, since they show an unusual fragmentation of the mass spectra of tretracyclic triterpenes containing the 9,19-cyclo-function (Figure 1) (Aplin and Horby, 1966; Audier et al., 1966).

The results in Table 1 clearly show chemical similarity of the labellar secretions of the three species investigated. A preliminary phylogenetic study of the

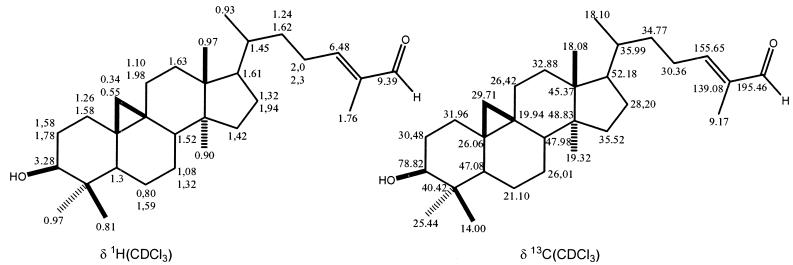

FIG. 2. Assignments of δ_H ($CDCl_3$) and δ_{C-13} ($CDCl_3$) of 3β-hydroxy-cycloart-24-en-26-al.

TABLE 1. COMPOSITION OF TRITERPENOID *Maxillaria cerifera*, *M. brasiliensis*, *M. friedrichsthalii* LABELLA REWARDS

Compound	Retention time	*m*/*z*	*M. cerifera* (%)	*M. brasiliensis* (%)	*M. friedrichsthalii* (%)
Cycloartenol derivative 1	13.55	426 (14%, M^+), 408 (35%), 393 (50%), 365 (24%), 339 (17%), 315 (4%), 286 (3%), 203 (22%), 69 (100%)	10.0	—	—
Cycloartenol derivative 2	13.75	426 (14%, M^+), 408 (35%), 393 (50%), 365 (24%), 339 (17%), 315 (4%), 286 (3%), 203 (22%), 69 (100%)	18.0	—	75.0
Cycloartenol derivative 3	13.77	426 (18%, M^+), 408 (36%), 393 (56%), 365 (28%), 339 (18%), 315 (5%), 286 (35%), 203 (20%), 69 (100%)	—	100.0	—
Cycloartenol derivative 4	14.30	440 (14%, M^+), 422 (34%), 407 (69%), 393 (4%), 379 (28%), 353 (10%), 300 (28%), 69 (97%), 55 (100%)	4.0	—	—
Cycloartenol derivative 5	16.45	440 (12%, M^+), 423 (40%), 410 (8%), 395 (6%), 379 (4%), 353 (10%), 300 (38%), 175 (32%), 95 (76%), 55 (100%)	—	—	25.0
3β-Hydroxy-cycloart-24-en-26-al	16.80	440 (6%, M^+), 422 (31%), 407 (53%), 379 (35%), 353 (18%), 300 (29%), 203 (34%), 95 (91%), 55 (100%)	68.0	—	—

Maxillariinae (Williams and Whitten, 2001) showed that species of *Maxillaria* that produce labellar wax-like secretions belong to two distinct clades. One of them includes *M. friedrichstahlii* and *M. cerifera* and related species, the other includes *M. brasiliensis* and related species. Thus, our results indicate that the chemical composition of labellar secretions in *M. friedrichstahlii* and *M. cerifera* is similar depicting cycloartenol derivative 2 that is absent in the labellar secretion of *M. brasiliensis.* The labellar secretions of the latter species is composed of cycloartenol derivative 3, which is absent from the secretions of the other two species. It would be interesting to investigate the chemical composition of the labellar secretions of other species of both clades to see if a stable pattern exists or not. Conclusive evidence is needed, but it seems that the *Maxillaria cerifera* floral reward is gathered by Meliponini bees.

Fragrance Analysis. Most of the rewarding Maxillariinae species previously studied lack detectable fragrance production (at least according to the human nose). A remarkable exception is *M. rufescens* and related species (such as *M. acutifolia* Lindl.). These latter species offer trichomes (whose chemistry is currently under study) as a reward and emit strong vanilla or chocolate-like fragrances. The flowers of several rewardless Maxillariinae species emit strong fragrances, which are largely responsible for long-distance pollinator attraction. Observations of pollination biology (Singer and Cocucci, 1999) indicate that in some cases the fragrances attract food-seeking hymenoptera. A survey of the chemical composition of the volatiles of seven Maxillariinae (Table 2) revealed a predominance of terpenes (mono and sesquiterpenes) with aldehydes and hydrocarbons, and their aromatic and fatty acid derivatives among the minor components. *M. gracilis* and *M. marginata* show similar fragrance compositions. The presence of 5-methyl-3-heptanone could be associated with pheromonal activity. Pollinator behavior suggests that the bees may visit these orchids in search of food sources. Differences in chemical composition between the fragrances of the two *Trigonidium* species suggest that different pollinators may be involved. The chemical composition of the volatiles of *T. obtusum* flowers (that attract male meliponine bees trying to copulate with flower parts) is simple, with pentadecane being predominant. Either the compound responsible for the sexual attraction is present in small amounts, or the active compound is coeluting with the pentadecane. So far, pentadecane itself failed to elicit any response from the bees during field tests. The fragrance of *M. jenischiana* is intense and unpleasant. Analyses indicated that 3-methylthio-1-hexanol may be responsible for the unpleasant aroma.

In summary, this report complements information on the chemical composition of *Maxillaria* labellar secretions, here named rewards, previously suggested as lipoidal by Davies et al. (2003). We were successful in determining their triterpenoid nature as well as in isolating cycloartenal, an unusual triterpene, together with cycloartenol derivatives. The results encourage further investigation to determine the importance of these materials in the lives of pollinating bees.

TABLE 2. VOLATILE COMPONENTS OF SOME MAXILLARIINAE, PERCENTAGE AND RETENTION INDICES DETERMINED BY GC/MS

Compound	RI calculated	RI literature	*M. gracilis* (%)	*M. marginata* (%)	*T.* cf. *turbinatum* (%)	*M. rufescens* (%)	*M. picta* (%)	*M. jenischiana* (%)	*T. obtusum* (%)
α-Pinene	938	939	—	0.4	—	—	5.8	—	—
5-Methyl-3-heptanone	943	943	0.7	0.5	—	—	—	—	—
Benzaldehyde	960	961	—	—	—	5.3	—	—	—
β-Pinene	983	981	—	—	1.0	—	—	—	—
6-Methyl-5-hepten-2-one	989	985	1.3	2.6	7.6	4.0	1.1	2.4	—
Mesitylene	1006	994	0.8	0.8	1.0	—	—	—	—
n-Octanal	1005	1001	—	—	3.6	—	—	0.4	—
p-Cymene	1032	1026	0.2	—	0.6	—	—	0.5	—
Limonene	1036	1031	0.2	9.0	6.8	—	—	—	—
cis-β-Ocimene	1045	1040	0.2	—	—	—	—	—	—
trans-β-Ocimene	1054	1050	30.3	1.3	0.5	—	—	—	—
Acetophenone	1073	1065	0.3	0.3	0.4	—	—	0.3	—
trans-Linalool oxide	1091	1088	0.3	0.5	—	—	—	—	—
Linalool	1102	1098	2.8	0.2	—	—	70.5	—	—
n-Nonanal	1106	1098	3.0	1.9	11.1	2.4	—	3.6	—
Phenyl ethyl alcohol	1113	1110	—	—	—	—	—	0.2	—
Menthol	1170	1173	—	—	—	—	—	0.3	—
Naphtalene	1182	1179	3.0	2.9	0.5	—	—	—	—
Methyl salicylate	1196	1190	—	0.6	—	—	—	—	—
Dodecane	1200	1200	—	0.8	—	—	—	—	—
3-Methylthio-1-hexanol	1203	1205	—	—	—	—	—	0.5	—
n-Decanal	1206	1204	4.9	4.4	18.3	21.8	2.6	10.0	—
p-Anisaldehyde	1252	1252	—	—	—	—	—	8.6	—
δ-Elemene	1338	1339	0.1	0.1	0.1	—	—	0.1	—
α-Cubebene	1350	1351	0.6	—	0.1	—	—	0.2	—
Cyclosativene	1368	1368	—	—	—	—	—	—	—
α-Ylangene	1371	1372	0.2	—	0.4	—	2.0	0.2	—
α-Copaene	1375	1376	5.7	9.6	11.0	13.0	—	13.6	—
β-Elemene	1392	1391	0.2	0.6	0.4	0.5	—	0.8	—

TABLE 2. (*Continued.*)

Compound	RI calculated	RI literature	*M. gracilis* (%)	*M. marginata* (%)	*T.* cf. *turbinatum* (%)	*M. rufescens* (%)	*M. picta* (%)	*M. jenischiana* (%)	*T. obtusum* (%)
n-Tetradecane	1400	1399	0.7	0.9	1.1	—	—	0.1	—
α-Gurjenene	1410	1409	0.1	—	—	—	—	0.1	—
cis-α-Bergamotene	1416	1415	0.3	0.4	0.4	0.7	1.1	1.0	—
trans-Caryophyllene	1419	1418	0.1	0.4	—	—	—	0.1	—
β-Gurjunene	1432	1432	0.1	0.2	—	—	—	0.2	—
Dihydro-β-ionone*	1436	—	—	—	—	—	9.6	—	—
Aromadendrene	1440	1439	0.1	0.1	—	2.8	—	0.2	—
α-Guaiene	1441	1439	—	—	—	—	—	0.2	—
Khusimene	1451	1447	—	0.1	—	—	—	—	—
α-Humulene	1453	1454	0.1	—	—	—	—	—	—
Geranyl acetone	1456	1453	0.9	1.5	—	5.5	—	0.3	—
Alloaromadendrene	1461	1461	1.0	2.0	1.7	—	—	3.6	—
γ-Muurolene	1477	1477	0.6	1.0	1.0	—	—	0.1	—
Germacrene D	1480	1480	0.2	1.0	1.1	1.2	—	—	—
ar-Curcumene	1484	1483	—	1.9	1.6	5.1	—	3.0	—
trans-β-Ionone	1483	1485	—	—	0.3	—	—	0.1	—
α-Selinene	1495	1494	0.1	0.3	—	—	—	0.7	—
α-Muurolene	1501	1499	1.7	2.0	2.8	2.9	—	4.6	—
Pentadecane	1500	1500	—	—	—	—	—	—	100.0
(*E*, *E*)-α-Farnesene	1510	1508	—	0.6	—	—	—	—	—
Butylated hydroxytoluene	1514	1512	2.6	17.7	2.5	—	—	5.3	—
δ-Cadinene	1524	1524	9.7	11.6	12.0	28.0	1.1	18.6	—
Cubebene	1531	1532	—	—	—	—	—	0.1	—
α-Calacorene	1544	1542	0.4	0.6	0.8	1.6	—	1.7	—
1-*epi*-Cubenol	1628	1627	0.2	0.1	0.1		—	—	—
Cadalene	1675	1674	—	—	—	—	1.4	—	—
Total identified	—	—	73.7	79.9	89.9	94.8	95.2	81.7	100.0
Not Identified	—	—	26.3	20.1	10.1	5.2	4.8	18.3	—

More research is needed concerning the fragrance composition of the rewardless Maxillariinae species. It is likely that some compounds may function as pheromones. Some rewardless Maxillariinae may simply be "food-fraud" flowers. In other species, refined mechanisms of mimicry are apparent. We hope that further work will allow a more accurate understanding of how different pollination strategies arose in the subtribe Maxillariinae.

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HUMIC SUBSTANCES AFFECT THE ACTIVITY OF CHLOROPHYLLASE

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Abstract—Three humic substances—humic acid, fulvic acid, and humin—were isolated from soils located in the northern and southern forests of the Yuanyang Lake Nature Preserve in northern Taiwan's Ilan County. Aqueous extracts of fresh wet soil and of three humic substances, at concentrations of 0.125, 0.25, and 0.5 mg/ml, were investigated for their effects on the activities of chlorophyllase a and b. Aqueous extracts of forest soils at the northern and southern bank, dominated by the pure vegetation of Formosan False cypress (*Chamaecyparis formosensis* Matsum), stimulate both chlorophyllase a and b activities, while those of the southern bank, dominated by a Taiwanese Miscanthus (*Miscanthus transmorrisonensis* Hayata), inhibits such activities. All three humic substances, despite their soil sources, stimulate the activities of both chlorophyllase a and b. Fulvic acid stimulates more chlorophyllase a activity than either humic acid or humin. Humic acid stimulates more activity of chlorophyllase b than either fulvic acid or humin. Humin exhibited the least effect on chlorophyllase a and b. It is suggested that humic substances in the soil may accelerate the chlorophyll degradation of litter in the ecosystem and that chlorophyllase a and b may be different enzymes.

Key Words—Humic substances, chlorophyllase, humic acid, fulvic acid, humin, chlorophyll degradation.

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INTRODUCTION

Humic substances affect the content and characteristics of soil organic matter and hence play an important role in the structure and function of soil (Schnitzer, 1991). The chemical structure of humic substances is complicated and not well known. However, the main structure of humic substances is a polycyclic aromatic core linked with side chain structures such as carbohydrates phenolics, proteins, peptides, and metal ions (Cheshire et al., 1967).

Research has confirmed that humic substances can indirectly and directly affect the physiological processes of plant growth. They provide minerals (Rauthan and Schnitzer, 1981), increase the microorganism population (Visser, 1985), provide biochemical substances (Vaughan and Malcolm, 1979), and carry trace elements and growth-related regulators (Chen and Schnitzer, 1978). Direct effects include increases in cell membrane permeability (Samson and Visser, 1989), respiration, nucleic acid biosynthesis, ion absorption (Vaughan et al., 1985), enzyme activity, and hormone-like activity (Albuzio et al., 1989; Piccolo et al., 1992; Nardi et al., 1994). Ladd and Bulter (1971) showed that humic acid inhibits the activities of carboxypeptidase A, pronase, chymotrypsin A, and trypsin, stimulates the activities of papain and subtilopeptidase, and shows no effect on phaseolain or tyrosinase. Malcolm and Vaughan (1979) demonstrated that humic acid inhibits the activity of invertase in mungbean hypocotyl and barley, but stimulates such activity in pea root. Ferretti et al. (1991) showed that low molecular humic acid can affect the activity of sulphate assimilation enzymes in corn, such as ATP-sulphurylase and *o*-acetylserine sulphydrilase, thus, promoting the use of sulphate. Liu et al. (1998) evaluated the effect of a commercial preparation of humic acid on the photosynthesis, chlorophyll concentration, and root development of creeping bentgrass and reported that chlorophyll content was unaffected by humic acid.

In another study, humic substances increased the protein content in leaf, but apparently decreased the chlorophyll content (Ferretti et al., 1991). It is unknown how humic substances decrease chlorophyll accumulation. They may inhibit the biosynthetic pathway of chlorophyll, stimulate the degradative pathway of chlorophyll, or both (Yang et al., 2002). This reduces chlorophyll accumulation and photosynthesis, which, in turn, diminishes total plant growth.

Chlorophyllase (chlorophyll-chlorophyllide hydrolase, EC 3.1.1.14) is an enzyme catalyzing the hydrolysis of chlorophyll to chlorophyllide and phytol and is believed to be present in most photosynthetic membranes of higher plants and algae (Rüdiger and Schoch, 1988; Lambers et al., 1986). Evidence suggests that chlorophyllase is located in the envelope of the chloroplast (Matile et al., 1997). Although much research has focused on the biochemistry and physiology of chlorophyllase (Drazkiewicz, 1994; Abdel-Basset et al., 1995), a survey of the literature indicates that little or no information is available regarding its role in the ecosystem or its interaction with humic substances.

The goal of our study was to determine the effects of three humic substances, isolated from the Yuanyang Lake Nature Preserve of northern Taiwan, on chlorophyllase activity.

METHODS AND MATERIALS

Sampling Site. The Yuanyang Lake Nature Preserve is located in the northeastern mountains of Ilan County in northern Taiwan, at the uppermost head water of the Tahan River watershed, with an elevation of 1670–2432 m. The total area of the reserve is about 374 ha, of which the lake is about 3.6 ha and the marsh area encircling the lake about 2.2 ha. The surrounding hillsides are dominated by valuable cypress forest. The climate is classified as temperate heavy moist: the monthly average maximal and minimal temperatures are $15 - 5$ and $0 - 5^{\circ}$C, respectively, and there is 100% relative humidity the whole year. The soil of five sampling sites, three on the south bank and two at the north bank of Yuanyang Lake, were collected from below ground 0–30 cm, because that depth contains most of the organic layer of the soil. Two southern sites, designated S1 and S2, are dominated by *Miscanthus transmorrisonensis*. Two northern sites N1, N2, and a southern site S3, are dominated by pure vegetation of false cypress *Chamaecyparis formosensis*. The distance between all sampling sites at the south or north bank exceeds 150 m.

Aqueous Extract. Two hundred grams of fresh wet soil collected from the field were extracted with 400 ml distilled water at room temperature and shaken at 300 rpm, for 2 hr. The crude extract was filtered through Whatman No. 1 paper, and the filtrate was centrifuged at 6,000*g* for 15 min at room temperature. The supernatant was lyophilized to dryness and weighed.

Isolation of Humic Substances. Soil samples collected from the field were air-dried at room temperature, ground, and passed through a 2 mm mesh. Fulvic acid, humic acid, and humus were isolated with 0.1 M HCl and 0.1 M NaOH, and purified according to the standard procedure of the International Humic Substance Society (Aiken, 1985; Hayes, 1985; Leenheer, 1985; Swift, 1985).

Preparation of Substrates. Chlorophyll a and b were isolated and purified from fresh leaves of spinach by extraction with cold acetone followed by column chromatography on a Pharmacia Biotech DEAE-Sepharose CL-6B (Lot No. 242436) and Sepharose CL-6B (Lot No. 230876) according to Omata and Murata (1983). The purified chlorophyll a and b were aliquoted, dried, wrapped with aluminum foil, and stored at –20°C until use.

Preparation of Acetone Powder. The liquid-nitrogen frozen leaf of an angiosperm pachira chestnut (*Pachira macrocarpa*) was ground with a motar and pestle, and then homogenized with prechilled acetone (–20°C). After centrifuging at 3000*g*, 4°C for 5 min, the precipitate was collected. The above procedure was repeated several times to remove as many trace chlorophylls and carotenoids as

possible. The acetone powder of *P. macrocarpa* leaf, abundant in the activity of chlorophyllase, was dried with nitrogen and stored at –20°C until use.

Assay of Chlorophyllase Activity. For the determination of chlorophyllase a and b activity, 50 mg of acetone powder of *P. macrocarpa* leaf was homogenized with 5 ml extraction buffer, containing 5 mM potassium phosphate (pH 7.0), 50 mM KCl, and 0.24% Triton X-100 for 60 min at 30°C. The supernatant, after centrifugation at 15,000*g* for 15 min, was used for the enzyme assay. The chlorophyllase assay followed a modified method of McFeeters et al. (1971). The standard reaction mixture contained 0.2 ml of substrate (1 μmol/ml chlorophyll a or chlorophyll b), 0.3 ml of the above supernatant, and 2 ml of reaction buffer containing 100 mM sodium phosphate (pH 7.0) and 0.24% Triton X-100, in the absence or presence of 0.125, 0.25, and 0.5 mg/ml humin, humic acid, or fulvic acid. The mixture was incubated for 30 min at 30°C, and the reaction was stopped with 0.5 ml of 10 mM KOH. After reaction, 1 ml of the reaction mixture was further mixed with 5 ml of hexane/acetone (3:2, v/v) to eliminate the interference of chlorophyll. The product chlorophyllide a or b in the acetone phase was determined by an Hitachi U-2000 spectrophotometer using an extinction coefficient of 74.9 mM^{-1} cm^{-1} at 667 nm or 47.2 mM^{-1} cm^{-1} at 650 nm for chlorophyllide a or b, respectively. One unit of chlorophyllase a or b was defined as the amount of enzyme needed to catalyze the production of 1 μM chlorophyllide a or b per min.

RESULTS

The aqueous extracts of soils from sites at the southern and northern bank show interesting contrasting effects on the activities of chlorophyllase a and b (Figure 1A and B). The aqueous extracts from the northern soil, N1 and N2, stimulated the activities of both chlorophyllase a and b, while those from the southern soil, S1 and S2, inhibited these activities. Various concentrations of northern bank extracts exhibited a more stimulatory effect on chlorophyllase b than on chlorophyllase a. In contrasts, the extract of the southern bank showed an inhibitory effect on the activities of both chlorophyllase a and b. The different effects result from locations, that are dominated by different vegetation. While the S1 and S2 sites are dominated by a *Miscanthus* grass community, N1 and N2 sites are dominated by false cypress. S3, located at the south bank, is also dominated by pure vegetation of false cypress, but its aqueous extract exhibited a stimulatory effect similar to that of N1 and N2.

While the chlorophyllase a/b ratio gradually declined as the concentrations of the extracts from sites N1, N2, and S3 increased, the ratio seemed to stay constant as extracts from sites S1 and S2 increased (Figure 1C). Aqueous extracts from the soils dominated by *Miscanthus* exhibited the same effect on chlorophyllase a and b as concentration increased, causing no change in the ratio. The aqueous

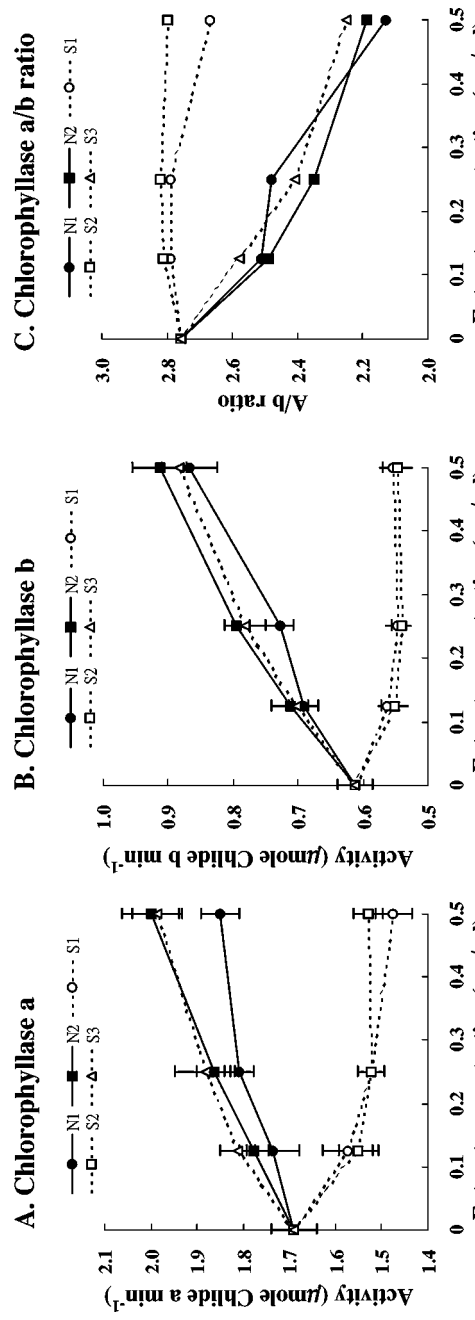

FIG. 1. The effect of aqueous extract from five forest soils at the southern and northern bank of Yuanyang Lake on the activities of chlorophyllase a (A) and b (B). Chlorophyllase a/b ratio (C) is the ratio of mean value of (A) and (B). Bars indicate the standard deviation of three determinations.

extracts from the soils dominated by false cypress showed an opposite effect on chlorophyllase a and b, with a decline of chlorophyllase a/b ratio.

Although the aqueous extracts from the two different vegetations exhibited opposite effects, all three humic substances isolated from all southern and northern sampling sites revealed stimulatory effects on chlorophyllase a and b activities to different degrees (Figure 2A and B). Fulvic acid showed a much more stimulatory effect on chlorophyllase a activity than humin or humic acid. Humic acid stimulated chlorophyllase b activity more than either humin or fulvic acid. For both activities, humin always showed the least effect among the three humic substances.

Interestingly, the three humic substances revealed three patterns of effect on the chlorophyllase a/b ratio, regardless of the soil they were isolated from (Figure 2C). The ratio of chlorophyllase a and b increased and then gradually decreased as the concentration of fulvic and humic acids increased. Humin showed the same effect on chlorophyllase a and b as its concentration increased, causing no change in the ratio. Fulvic and humic acids had opposite effects on chlorophyllase a and b, leading, respectively, to the increase and decrease of the chlorophyllase a/b ratio.

DISCUSSION

Chlorophyll degradation is a sequence of biochemical and physiological events comprising the final stage of development (Smart, 1994; Vicentini et al., 1995). It has been reported that irradiance, temperature, water, osmotic stresses, fertility, infections, plant age, and growth regulators can affect the activity of chlorophyllase (Drazkiewicz, 1994; Abdel-Basset et al, 1995). However, no report links how the humic substances of forest soil ecosystems affect the chlorophyll degradation machine of fallen leaves, whether senescent or fresh.

The contrasting effects on chlorophyllase a and b suggest that the aqueous extracts of soil from sites S1 and S2 contain unknown water-soluble compounds that inhibit both chlorophyllase a and b activities. Those compounds may be lost during the process of isolating the humic substances, causing the disappearance of any inhibitory effects. It appears that the aqueous extracts of soil from sites N1, N2, and S3 contain no inhibitiory compounds.

Stimulatory effects were found with all three humic substances, regardless of their soil source. Since vegetation in the five sampling sites is different, different plants probably contribute and release different amounts of organic matter, generating different quantities and qualities of humic substances in their corresponding soils, which, in turn, results in different effects on chlorophyllase activity. The different responses of chlorophyllase a and b to the three humic substances suggests that the interaction mechanism between the two chlorophyllases and humic substances may be different.

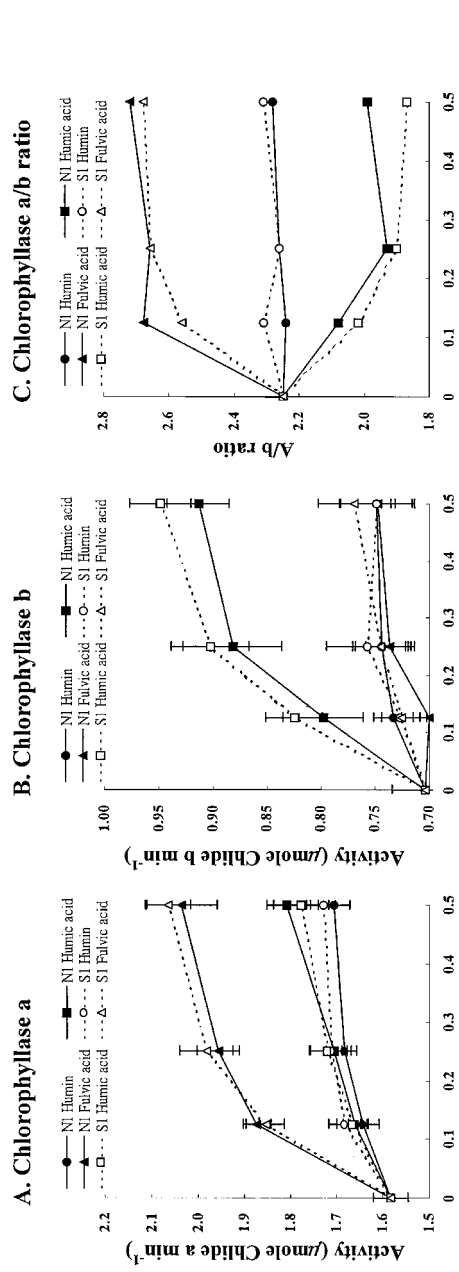

FIG. 2. The effect of three humic substances isolated from forest soils at the southern and northern bank of Yuanyang Lake on the activities of chlorophyllase a (A) and b (B). Chlorophyllase a/b ratio (C) is the ratio of mean value of (A) and (B). Bars indicate the standard deviation of three determinations.

Although humin had similar effects on chlorophyllase a and b, both fulvic and humic acids showed opposite effects. The ratio of chlorophyllase a to b activity is positively correlated with the concentration of fulvic acid, but inversely correlated to that of humic acid. These results suggest that chlorophyllase a and b are two different enzymes. Although individual humic substances affect the chlorophyllase a and b differently, the chlorophyll breakdown of fallen leaf in the forest ecosystem is, of course, a combination of effects of all humic substances in the soil. From an ecological standpoint, it is not realistic to think that humic substances in the soil can affect the chlorophyll biosynthetic pathway in a leaf, but they could affect chlorophyll degradation processes. Leaves, at all developmental stages, fall to the ground and either contact or get buried in soil or immersed in lake water containing a different quality and quantity of humic substances coming from different vegetation.

Phenolic acids are known to be important components of humic substances in soil (Cheshire et al., 1967; Stevenson, 1982). Some phenolic compounds, such as *o*-hydroxyphenylacetic, ferulic, and *p*-coumaric acid inhibit the activity of the chlorophyll biosynthetic enzyme Mg-chelatase (Yang et al., 2002), and significantly induce the activities of chlorophyllase a and b, and Mg-dechelatase a and b in rice seedlings (Yang et al., 2004). Thus, the stimulatory effect of humus on chlorophyllase activity may stem from phenolic compounds. Other components may also have effects on chlorophyllase activity. Our findings suggest the importance of further investigation to understand the nature of humic substances and their effects on the degradation of chlorophyll in relation to the nature of physiological ecology of carbon and nitrogen cycles in forest ecosystems.

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ALLELOCHEMICALS OF *Polygonella myriophylla*: CHEMISTRY AND SOIL DEGRADATION

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Abstract—Gallic acid and hydroquinone have been identified as the major allelochemicals of the known allelopathic plant *Polygonella myriophylla*. Both of these compounds occur in the foliage as glycosides. Quercetin and rhamnetin were identified as the major flavonoid constituents, but in much lower concentration. The behavior of gallic acid, hydroquinone, the hydroquinone glycoside arbutin, and benzoquinone in sterile and nonsterile soil from beneath *Polygonella* was investigated. Sterilization effectively stabilized arbutin, hydroquinone, and gallic acid. Concentrations of benzoquinone rapidly diminished in sterilized soil, and the compound was almost completely gone after 7 days. In nonsterile soils, all four compounds degraded rapidly. The order of persistence was hydroquinone > benzoquinone > gallic acid > arbutin. Persistence was rate-dependent. Arbutin degraded to hydroquinone, and benzoquinone formed as a degradation product of hydroquinone. Hydroquinone was also observed as a degradation product of benzoquinone. Benzoquinone degrades rapidly by nonmicrobial oxidative processes. These results support the hypothesis that microbial and nonmicrobial oxidative transformations of soil allelochemicals are crucial in mediating the allelopathic effects of *Polygonella myriophylla*.

Key Words—*Polygonella myriophylla*, allelopathy, arbutin, benzoquinone, gallic acid, hydroquinone, sand pine scrub.

INTRODUCTION

Polygonella myriophylla (Small) Horton is one of several shrubs endemic to the Florida sand pine scrub community found to inhibit the germination and growth of grasses from the neighboring Florida sandhills (Weidenhamer and Romeo, 1989). Striking bare zones surround mature *Polygonella* stands, and the scrub itself is

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characterized by distinct boundaries where it borders roads or the adjacent Florida sandhill community. The bare zones around mature shrubs and at these boundaries are typically 1 m or more in extent. Scrub vegetation is sensitive to fire, and it has been proposed that chemical interference by scrub perennials prevents the invasion of scrub by grasses and herbs that would otherwise provide fuel for fires that would kill these shrubs (Richardson and Williamson, 1988). Other scrub species for which there is evidence of allelopathic effects include *Ceratiola ericoides* Michx. (Tanrisever et al., 1987), *Conradina canescens* (Torr. & Gray) A. Gray (Williamson et al., 1989), *Calamintha ashei* (Weatherby) Shinners (Weidenhamer et al., 1994), and *Chrysoma pauciflosculosa* (Michx.) Greene (Menelaou et al., 1993). The extensive work on the role of allelopathic interference in the Florida scrub community has been the subject of several reviews (Fischer et al., 1994; Romeo and Weidenhamer, 1998).

Polygonella myriophylla is a prostrate, woody plant that forms dense mats with numerous primary and secondary branches. Previous work (Weidenhamer and Romeo, 1989) established that *Polygonella* is allelopathic toward bahiagrass (*Paspalum notatum*, a ruderal species) and little bluestem (*Schizachyrium scoparium*, a sandhill grass). Quantitative measurements of root mass distribution showed that few *Polygonella* roots extend into the bare zones, ruling out competition as a factor in maintaining the bare zones. Soil collected biweekly from beneath and around *Polygonella* was bioassayed with both grass species. The average germination of bahiagrass was 71% in *Polygonella* soil and 81% in bare zone soil, compared to soil from an adjacent grassed area. Average shoot dry weight of bahiagrass was 48% in *Polygonella* soil, and 81% in the bare zone.

Studies were done to identify the major allelochemicals in *Polygonella myriophylla*. Significant evidence from bioassays (Weidenhamer et al., 1989) and the literature (Chou and Muller, 1972; Manners and Galitz, 1985; Hogan and Manners, 1990) suggested the potential importance of two of these compounds, gallic acid and hydroquinone, as allelopathic agents. For this reason, experiments were carried out to determine the behavior of gallic acid, hydroquinone, the hydroquinone glycoside arbutin, and the hydroquinone oxidation product, benzoquinone, in sterile and nonsterile soil from beneath *Polygonella*.

METHODS AND MATERIALS

Phytochemical Investigation

Plant Material. A voucher specimen of *Polygonella myriophylla* was collected south of Sun Ray, Florida (Range 28E, Township 32S, NW Quarter of section 18) and deposited in the University of South Florida herbarium (no. 190013). Bulk samples of *Polygonella* foliage for chemical analysis were collected at several sites south of Lake Wales, Florida.

Foliar Extracts. Hydrolyzed extracts of *Polygonella* foliage were prepared according to Harborne (1984) to examine the phenolic aglycones present. Fresh or frozen leaves were covered with 2 M HCl in a small Erlenmeyer flask and placed in a boiling water bath for 30–40 min. After cooling, the aqueous phase was filtered through glass wool and extracted four times with a one-fourth volume of ethyl acetate. The ethyl acetate fraction was then concentrated by rotary evaporation for analysis. For samples being analyzed quantitatively, moisture content was determined on a 250–500 mg portion after 48 hr at 80°C. The major phenolics and flavonoids were isolated by preparative thin layer chromatography (TLC) on cellulose (described below).

Nonhydrolyzed foliar extracts were prepared to examine the phenolic glycosides that occur in *Polygonella* (Haslam, 1965). Fresh leaves (2.16 g) were soaked in 100 ml hexanes for 30 min. This solution was decanted and the leaves were chopped in a Waring blender for 3 min with 150 ml water chilled to 10°C. The resulting solution was centrifuged for 20 min at $10,000 \times g$. The supernatant was filtered, diluted to 300 ml, acidified with 1 ml of 2 M HCl, and extracted with ethyl acetate (1×200 ml, 3×75 ml). The ethyl acetate fraction was then concentrated by rotary evaporation.

Analysis. Samples were analyzed by TLC on silica and cellulose. Solvents for silica TLC were acetic acid: chloroform (1:9), ethyl acetate:benzene (45:55), and toluene:chloroform:acetone (40:25:35). Solvents for cellulose TLC included 6% acetic acid and chloroform:acetic acid:water (50:45:5). All solvent proportions are expressed as v:v. Compounds were detected by fluorescence under short- (254 nm) and long-wave (366 nm) ultraviolet light before and after fuming with ammonia; sprays of Folin reagent (Sigma Chemical Co., St. Louis, MO) and saturated potassium iodate to detect phenolic aglycones; and sprays of 1% Gibbs reagent (2,6-dichloroquinone-*N*-chloroimide; Sigma Chemical Co.) in methanol followed by saturated sodium bicarbonate to detect phenolic glycosides.

Ultraviolet spectra were measured on an IBM 9420 double beam spectrophotometer. For flavonoids, the analysis followed Mabry et al. (1970). Melting points were determined on a Fisher–Johns melting point apparatus. Infrared spectra were determined in Nujol mull on a Beckman 1100 infrared spectrophotometer. Proton NMR spectra were determined on a Jeol FX90Q NMR spectrometer. Trimethylsilyl ethers of *Polygonella* flavonoids were prepared to facilitate NMR analysis according to the procedure of Mabry et al. (1970). Quantitation of the identified phenolics and flavonoids was carried out on a Bio-Analytical Systems Inc. high-performance liquid chromatograph (HPLC), using an octyldecyl silyl (C18) column (150×4 mm) and detection at 254 nm. A Shimadzu Chromatopac C-3RA was used for peak integration and retention time measurements. A Rheodyne Type 50 Teflon low-pressure switching valve was used to elute compounds with a step gradient of methanol:water:acetic acid (2.5:97.5:1, v:v:v) for 3 min, followed by methanol:water:acetic acid (70:30:1). Solvent flow rates were 1.5 ml min^{-1}, and

samples were filtered through a 0.45 μm nylon syringe filter immediately before analysis.

Reagents. All solvents were reagent grade. Hydroquinone (Fisher Scientific Co., Fair Lawn, NJ), arbutin, gallic acid, and quercetin (all from Sigma Chemical Co., St. Louis, MO) were obtained as standards for chromatographic and spectral comparisons.

Soil Degradation Study

Soil Samples. Soil was obtained from the top 5 cm beneath mature *Polygonella* shrubs. Scrub soil typically has >90% sand, an acidic pH, and is very low in available nutrients (Weidenhamer and Romeo, 1989). Soil was air-dried immediately after collection and passed through a 1.5 mm screen to remove large debris. One gram samples were weighed into 15 $\times$ 125 mm test tubes. Sterilized soils were prepared in the same way, but autoclaved three times at 48 hr intervals (Dalton et al., 1989). It is recognized that air drying may have affected the microbial status of the soil. However, the scrub environment is one in which there is frequent wetting and drying due to periodic rainfall, rapid drainage of the sandy soil, and high temperatures that dry out the soil. For these reasons, it is believed that this procedure should not have had a large qualitative effect on the soil microflora.

Treatments. Phenolics were added to the soil in water sufficient to bring the moisture content to 12.5%. This is slightly below field capacity, and the same water content used in previous bioassays of *Polygonella* soil (Weidenhamer and Romeo, 1989). Treatments included a control, and three rates (100, 200, and 400 μg g^{-1} soil) of each of the four phenolics (arbutin, benzoquinone, gallic acid, and hydroquinone). Concentrations selected were in the range that effects were seen in previous bioassays with bahiagrass (Weidenhamer et al., 1989). Phenolic solutions were filter-sterilized into autoclaved glassware before addition to samples. Soil samples were sealed with Parafilm and stored in the dark at ambient temperature until extraction and analysis. Duplicate samples of each treatment were extracted at 1, 2, 3, 5, 7, and 9 days. A "time zero" extraction, to check immediate recovery, was made 1 hr after the initial spiking of samples.

Extraction Procedure. Water, methanol, and 0.25 M citrate were evaluated as possible extractants for preliminary experiments (Dalton et al., 1987; Blum, 1997). Citrate has been recommended over EDTA as a better extractant for recovering biologically meaningful (i.e., available to plants and microorganisms) concentrations of phenolics from soil (Blum, 1997). However, for these compounds and this soil, citrate does not appear to be useful. Water and methanol yielded equivalent recoveries of phenolics, while recovery of hydroquinone and gallic acid were significantly lower with 0.25 M citrate (data not presented). Peak shapes were better on HPLC with water, so water was used as the extractant for these studies.

Soil samples were extracted with 2 ml water for 60 min, during which time they were mixed by vortexing three times. The resulting extracts were briefly centrifuged and then filtered through 0.2 μm nylon filters before analysis. Samples not immediately analyzed were refrigerated for a maximum of 24 hr. No degradation of the filtered samples was observed over this time.

HPLC Analysis. The HPLC system included a Spectra Physics Model 8800 ternary gradient pump, manual Rheodyne injection valve, Microsorb MV (Rainin Instruments) octyldecylsilyl (C18) column (200 $\times$ 4 mm) and Dionex variable wavelength detector. A Varian 4270 integrator was used for peak integration and retention time measurements. Compounds were eluted isocratically with water/methanol/acetic acid (97.9/2.0/0.1, v/v/v) at 1.0 ml min^{-1}. Solvents were HPLC grade. Injection volume was 10 μl. Detection wavelengths were 294 nm (hydroquinone and arbutin), 272 nm (gallic acid), and 242 nm (benzoquinone). Detection limits were approximately 1 μg g^{-1} soil for each compound.

RESULTS

Phytochemical Investigation

More than 35 distinct components were evident in the hydrolyzed *Polygonella* extracts by two-dimensional TLC on cellulose. The major phenolic compounds in the extract, as evidenced by the intensity of reaction with Folin reagent, were gallic acid, hydroquinone, and an unidentified phenol. The identities of gallic acid and hydroquinone were confirmed by comparison of their chromatographic and spectral properties to known standards. The major flavonoid aglycones, indicated by their intense yellow fluorescence under ultraviolet light, were quercetin and rhamnetin. The identity of quercetin was confirmed by comparison of its chromatographic and spectral properties to standard material, while rhamnetin was confirmed by comparison of its spectral data to that reported by Mabry et al. (1970).

The aglycones of gallic acid, hydroquinone, quercetin, and rhamnetin were not detected in nonhydrolyzed extracts of *Polygonella* foliage. Eight major glycosides were found, including three flavonol glycosides, two galloyl esters, two hydroquinoyl-galloyl esters, and arbutin (hydroquinone-β-D-glucoside). Flavonol glycosides were characterized by an intense yellow fluorescence after fuming with ammonia. Galloyl esters were identified by means of their positive reaction with potassium iodate sprays and formation of a brown to purple color with Gibbs reagent (Britton and Haslam, 1965; Haslam, 1965; Haddock et al., 1982). Hydroquinoyl esters, including arbutin, gave an intense sky-blue coloration on treatment with Gibbs reagent (Haslam et al., 1964; Britton and Haslam, 1965). Arbutin was identified by comparison of its chromatographic and spectral properties to the known standard. One of the hydroquinoyl-galloyl esters co-chromatographed

TABLE 1. CONCENTRATIONS OF GALLIC ACID (GA), HYDROQUINONE (HQ), QUERCETIN (QU), AND RHAMNETIN (RH) IN FOLIAGE AND LITTER OF *Polygonella Myriophylla* AND OTHER *Polygonella* SPP. AS DETERMINED BY HPLC

	Concentration, mg g^{-1} dry weight (±SE)			Concentration, ng g^{-1} dry weight (±SE)	
	GA	HQ	GA + HQ	Qu	Rh
Sampling date					
December 21, 1985	1.5 ± 0.1	3.75 ± 0.05	5.25 ± 0.15	4.15 ± 1.05	21.1 ± 20.8
March 18, 1986	2.3 ± 0.2	4.35 ± 0.35	6.65 ± 0.15	9.95 ± 1.05	25.9 ± 0.8
June 21, 1986	2.05 ± 0.25	4.05 ± 0.25	6.1 ± 0.0	5.85 ± 0.65	9.5 ± 1.2
September 12, 1986	1.6 ± 0.0	3.6 ± 0.1	5.2 ± 0.1	4.6 ± 1.9	10.4 ± 0.4
December 4, 1985 (litter)	0.2	ND	0.2	ND	ND
May 28, 1986 (litter)	0.6	ND	0.6	ND	ND
Other species					
P. ciliata var. *basiramia*	0.45	ND[a]	0.45	1.2	ND
P. fimbriata	4.3	0.7	5.0	ND	ND
P. polygama	5.3	0.3	5.6	> 40	ND

Note. Values for foliage of *P. myriophylla* are the mean of two samples, and standard errors are shown. Samples of other *Polygonella* spp. were obtained from field specimens of these plants.

[a] ND: Not detected. Approximate detection limits were 0.01 mg g^{-1} GA, 0.3 mg g^{-1} HQ, and 0.3 ng g^{-1} Qu and Rh.

with 2-O-galloyl arbutin obtained from an herbarium specimen of *Arctostaphylos uva-ursi*. The other glycosides have not yet been characterized.

Concentrations of gallic acid and hydroquinone in *Polygonella* were 5–6 mg g^{-1} dry weight (Table 1). Concentrations of hydroquinone were approximately twice that of gallic acid. While rhamnetin was the major flavonoid (concentrations of 10–26 ng g^{-1} compared to 5–10 ng g^{-1} of quercetin), flavonoid concentrations were much lower than the concentrations of the phenolics. Interestingly, concentrations of hydroquinone were much lower in samples of three other *Polygonella* species tested. All three occur in the scrub, but of these only *Polygonella fimbriata* has patterns of distribution suggesting a possible allelopathic effect. Leaf washes of *Polygonella myriophylla* (data not shown) showed the presence of the glycosides, but not the aglycones of these four compounds.

Soil Degradation Study

Sterile Soil. Sterilization effectively stabilized arbutin, hydroquinone, and gallic acid (Figures 1a, c, and 2a). Recovery of arbutin, hydroquinone, and gallic acid after 9 days, averaged across rates, was 99.7, 72.6, and 72.9%, respectively. Low concentrations of benzoquinone (< 5 μg g^{-1}) were observed in soil 5–9

days after treatment with hydroquinone (Figure 2b). Benzoquinone behaved quite differently. Concentrations of benzoquinone diminished rapidly, and were almost completely gone from the soil after 7 days (Figure 3a). A portion, but not all, of this benzoquinone was reduced to hydroquinone, which then persisted through the course of the experiment (Figure 3b).

Nonsterile Soil. In unsterilized soil, all four compounds rapidly degraded (Figures 1b, d, 2c, and 3c). Hydroquinone was the most persistent, followed by benzoquinone. Gallic acid was less persistent, reaching nondetectable levels after 3 days, even at the highest applied rate (Figure 1d). Arbutin was the least persistent, and reached nondetectable levels after 2 days at all applied rates (Figure 1b). Persistence of all compounds was rate-dependent, with higher rates persisting for longer periods of time.

Arbutin degraded to hydroquinone as expected (Figure 1b). At the 400 μg g^{-1} rate, complete conversion of arbutin to hydroquinone would result in a corresponding hydroquinone concentration of 162 μg g^{-1}. Instead, a maximum recovery of 82 μg g^{-1} hydroquinone was observed. At this rate, concentrations of hydroquinone remained in the range of 70–80 μg g^{-1} for 1–3 days after treatment. Concentrations of hydroquinone reached negligible levels after 5 days at all applied rates of arbutin.

Benzoquinone did form as a degradation product of hydroquinone (Figure 2d). Interestingly, at the highest rate of hydroquinone, the resulting benzoquinone persisted through the end of the experiment, while in benzoquinone-treated soils, benzoquinone disappeared by 9 days at all applied rates (Figure 3c). The reason for this is unclear. Perhaps surprisingly, some hydroquinone was formed as a degradation product of benzoquinone in both sterile and unsterilized soil (Figure 3b and d).

DISCUSSION

Manners and Galitz (1985) found arbutin in high yield (0.034% of dry plant mass) in *Antennaria microphylla*. They attributed the allelopathic effects of this plant toward leafy spurge to the breakdown of arbutin to hydroquinone and benzoquinone. The concentration of hydroquinone found in *Polygonella* tissue was as high as 0.435% on a dry plant mass basis (Table 1), which would correspond to a concentration of 1.1% arbutin if all of the hydroquinone were present as arbutin (which it is not). However, the presence of hydroquinone at concentrations more than 10 times that of another species in which it is thought to be the principal allelopathic agent suggests that this compound has a role in the observed allelopathic effects of *Polygonella myriophylla*.

Our results demonstrate that under these conditions, hydroquinone is rapidly produced from arbutin in these soils as the result of microbial degradation. At the highest applied rate of arbutin, hydroquinone concentrations in the soil remained

relatively constant for 1–3 days, reflecting the dynamics of gradual formation from arbutin and subsequent degradation. Benzoquinone, which is more toxic than hydroquinone in comparative bioassays (Manners and Galitz, 1985), was observed as a degradation product of hydroquinone. This is the first demonstration

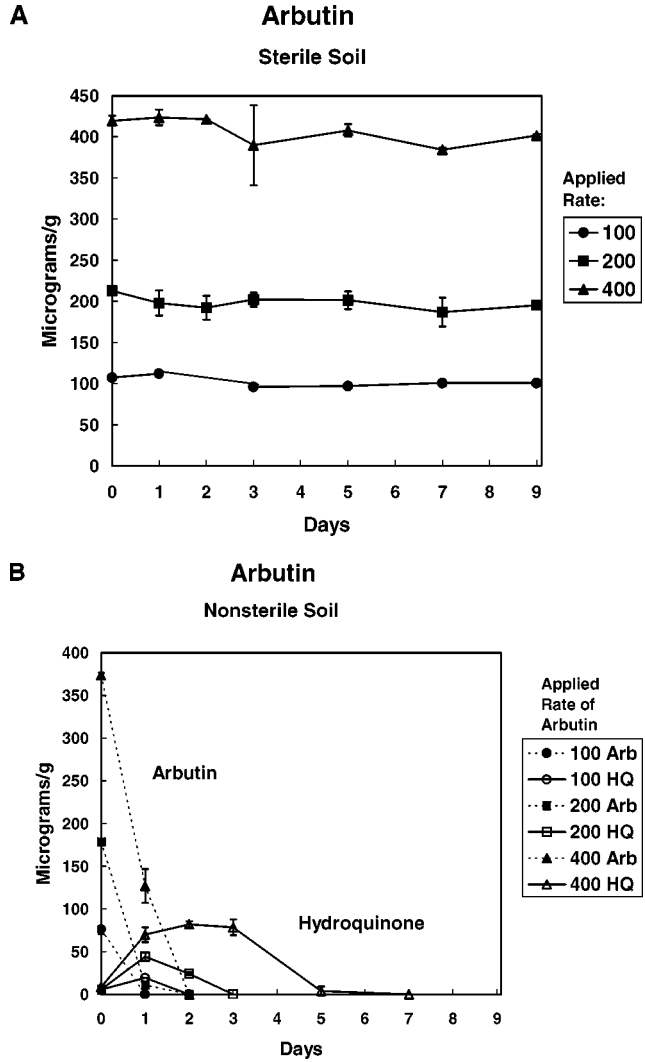

FIG. 1. Degradation of exogenously applied arbutin (Arb) and gallic acid (GA) in sterile and nonsterile soil. Bars indicate standard error. If not shown, standard errors are too small to depict visually. Hydroquinone (HQ) arose as a degradation product of arbutin.

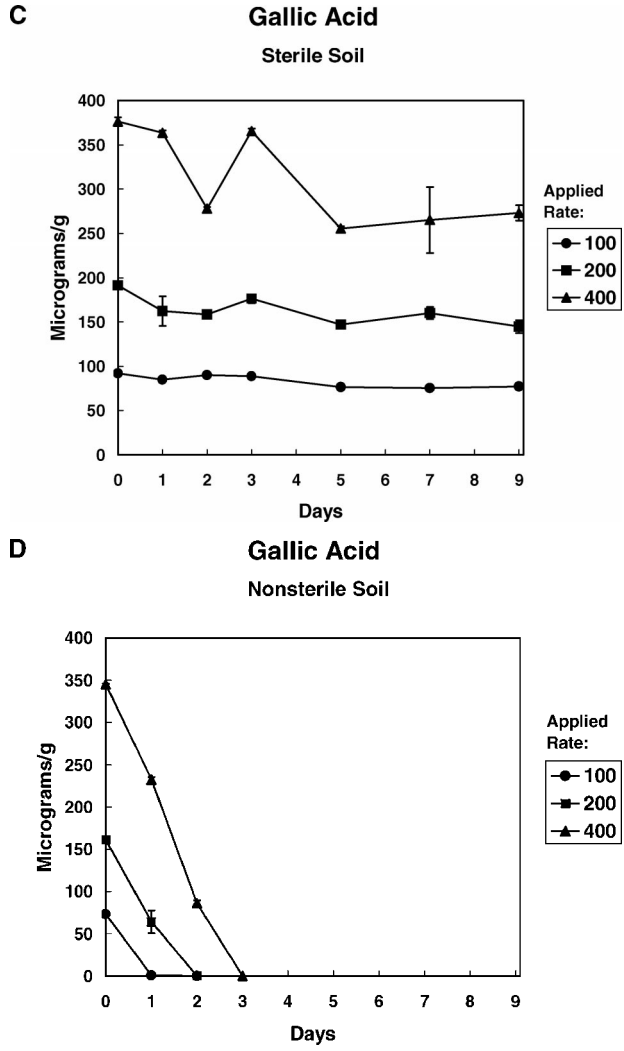

FIG. 1. (Continued)

that we are aware of that shows benzoquinone production in soils receiving arbutin or hydroquinone. Investigation of the effects of benzoquinone on grasses known to be inhibited by *Polygonella* would be warranted, on the basis of our results.

Given the susceptibility of both gallic acid and hydroquinone to chemical oxidation (Flaig et al., 1963; Tulyathan et al., 1989), it was surprising that little or no degradation of these compounds was observed in sterile soil. In contrast, the

rapid disappearance of benzoquinone even in sterilized soils suggests that chemical oxidative processes may be more important for some hydroquinone breakdown products than others. Previous bioassays have shown that the toxic effects of hydroquinone and gallic acid persist for several weeks after these compounds have

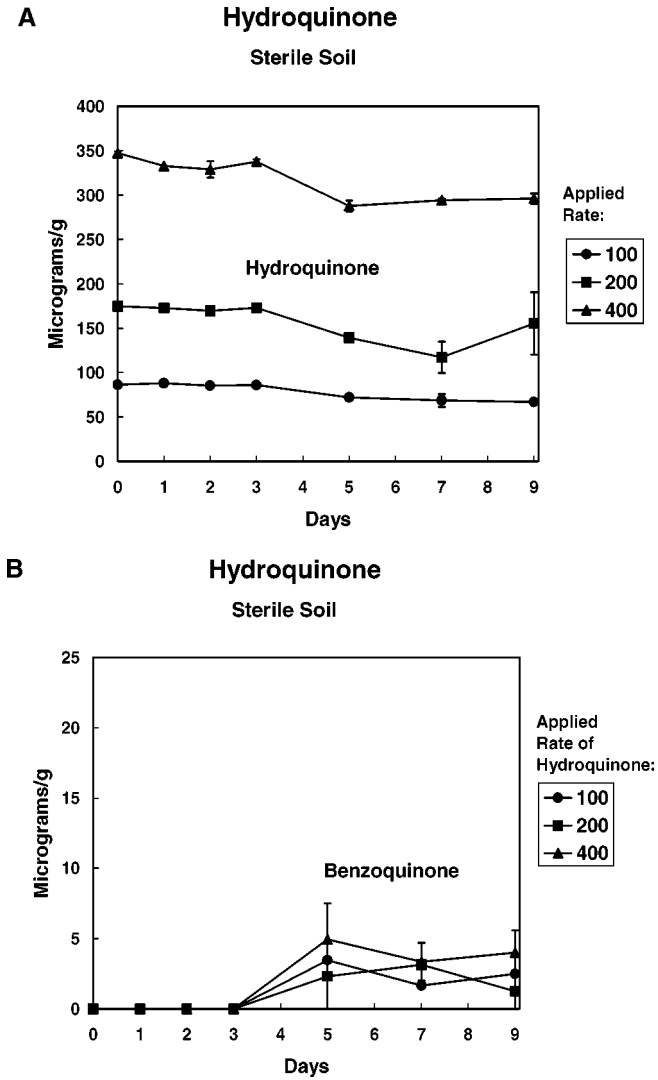

FIG. 2. Degradation of exogenously applied hydroquinone (HQ) in sterile and nonsterile soil. Bars indicate standard error. If not shown, standard errors are too small to depict visually. Benzoquinone (BQ) arose as a degradation product of hydroquinone.

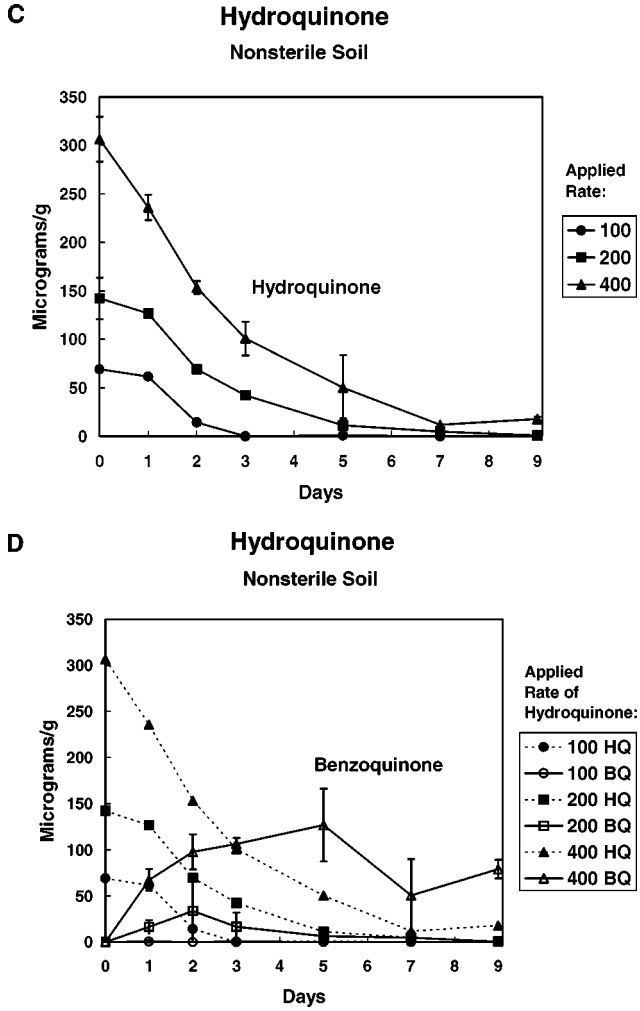

FIG. 2. (Continued)

disappeared from the soil (Weidenhamer et al., 1989). This suggests that the observed toxicity is due to as yet uncharacterized microbial and/or chemical oxidation products of gallic acid and hydroquinone/benzoquinone.

The importance of microorganisms in the mitigation and mediation of allelopathic effects has been emphasized by many investigators (Kaminsky, 1981; Blum and Shafer, 1988; Schmidt, 1988; Shafer and Blum, 1991; Pue et al., 1995; Blum, 1998; Blum et al., 1999). Our results demonstrate the importance of microorganisms in both activating and degrading allelochemicals from *Polygonella*. While

Schmidt (1988) argued that the presence of microorganisms adapted to degrading juglone was evidence that juglone was not allelopathic, Williamson and Weidenhamer (1990) argued that plant roots as well as microorganisms will compete for toxins in the soil, and that the outcome of this competition might determine whether or not allelopathic effects occur. Our current hypothesis is that microbial and nonmicrobial oxidative transformations of *Polygonella* allelochemicals

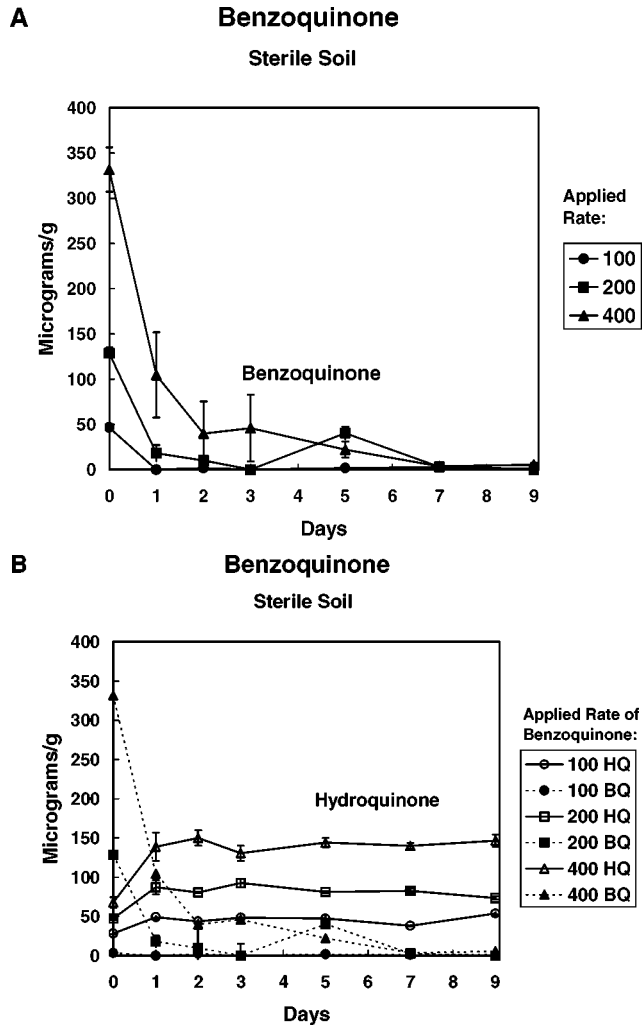

FIG. 3. Degradation of exogenously applied benzoquinone (BQ) in sterile and nonsterile soil. Bars indicate standard error. If not shown, standard errors are too small to depict visually. Hydroquinone (HQ) arose as a degradation product of hydroquinone.

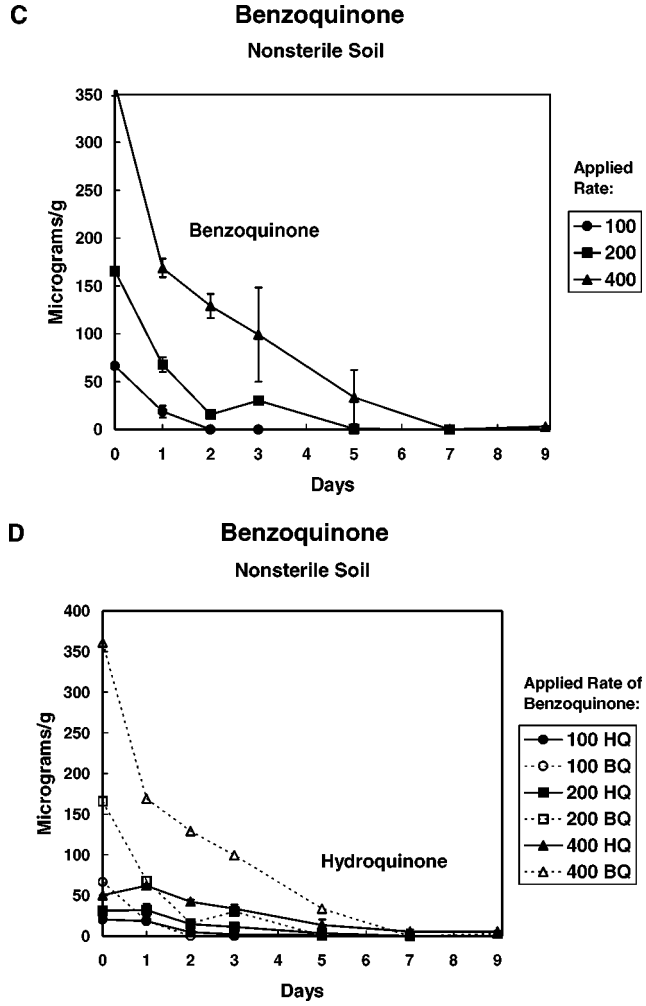

FIG. 3. (Continued)

are crucial in mediating the allelopathic effects of *Polygonella myriophylla*. The extent to which gallic acid, hydroquinone, and their derivatives are involved in the observed effects remains to be conclusively demonstrated. The toxicity of *Polygonella* allelochemicals and their breakdown products is likely exacerbated by the harsh environmental conditions in the scrub, including high temperature, nutrient limitation, and periodic moisture stress (Williamson et al., 1992; Weidenhamer, 1996). We recognize that the ambient laboratory temperatures of these studies differ from the temperature regimes in the Florida scrub, where sur-

face soil temperatures can exceed 50°C on summer days. It would be useful to repeat these investigations using a temperature regime more similar to the scrub environment in order to get a more accurate determination of residence times for these compounds.

Finally, this study raises questions about bioassay-guided fractionation, which has been used to identify potential allelopathic agents in numerous species (Lehle and Putnam, 1983). In the case of *Polygonella myriophylla*, our hypothesis is now that chronic low doses of hydroquinone and/or benzoquinone are the principle phytotoxins responsible for the observed allelopathic effects. Neither compound occurs in the plant itself, with hydroquinone being produced by microbial breakdown of arbutin (and presumably other hydroquinone-containing glycosides), and benzoquinone being produced by the degradation of hydroquinone. Environmental transformation of plant allelochemicals has been found to be important for two other scrub perennials. Leaf washes of *Ceratiola ericoides* contain the dihydrochalcone ceratiolin. This compound, which is not very active, degrades in aqueous solution into the much more phytotoxic hydrocinnamic acid on exposure to light (Tanrisever et al., 1987; Fischer et al., 1994). Leaf washes of *Chrysoma pauciflosculosa* were found to contain the diterpene 17-hydroxygrindelic acid as a major component. This compound readily oxidizes to the more toxic 17-oxogrindelic acid (Menelaou et al., 1993). Collectively, these results suggest that bioassay-guided fractionation of plant extracts may be inadequate for studies of allelopathy. If evidence from bioassays suggests that environmental and/or microbial transformation may be important, it may be necessary to fractionate extracts that are exposed to environmental conditions and/or soil microorganisms.

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Book Review

Allelopathy: Chemistry and Mode of Action of Allelochemicals. Edited by Francisco A. Macías, Juan C. G. Galindo, José M. G. Molinillo, and Horace G. Cutler. CRC Press, Boca Raton, Florida, 2004. $129.95, £87.00. ISBN 0-8493-1964-1

Allelopathy is the study of chemically mediated interactions between plants. Given the recent pace of discovery in this field, publication of a volume on the chemistry of allelopathy is timely. In the preface, the editors suggest that the development of the science of allelopathy has not been unlike the development of a painting, which may begin with sketches and outlines but progresses to a highly detailed and substantive work of art. This volume demonstrates how far research in this field has come since Alan Putnam and C. S. Tang wrote in 1986 that "chemistry has been the Achilles' heel of allelopathy." Clearly this is no longer the case, and work being done is providing a much more detailed picture of the mechanisms of chemical interactions between plants. Chapters focus on the chemistry of allelopathic interactions, including detailed structure–activity work, and on the mode of action of allelochemicals. In general, the chapters are well written and provide helpful bibliographies that contain links to both classic and newer literature.

Chapters on chemistry begin with a review of the activity of diterpenes isolated from the Potamogetonaceae on aquatic organisms by M. DellaGreca et al. There is a strong applied orientation throughout the volume, and several chapters focus on the chemistry of agricultural crops. D. Sicker et al. cover the chemistry and effects of benzoxazolin-2(3*H*)-ones, compounds that occur in rye, wheat, and maize. F. A. Macías et al. present a study of the structure–activity relationships for a number of heliannanes, a new class of sesquiterpenes isolated from sunflower (*Helianthus annuus*). This chapter is one of numerous instances throughout the text in which phytotoxicity is really the subject rather than allelopathy. By and large, however, the phytotoxicity assays are being used as tools to address meaningful questions, as evidence by this chapter in which the stated goal is a search for natural herbicides. J. C. G. Galindo et al. provide a solid review of the fascinating chemical signaling involved in the germination of the parasitic witchweeds (*Striga* spp.) and broomrapes (*Orobanche* spp.). They report a detailed structure–activity study with derivatives of strigolactones tested against both witchweed and broomrape.

Phenolic acids continue to attract the interest of researchers in this field, and three chapters review the chemistry and mode of action of phenolics. T. Haig

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demonstrates the power of modern analytical techniques by using GC–MS–MS to study wheat root exudates. Quantitative data on phenolic acids and the benzoxazolinone Dimboa are reported for 58 wheat accessions. A chapter by U. Blum summarizes the current state of knowledge on the role of microorganisms in metabolizing phenolics in soil. This insightful discussion points out numerous lines of needed research. F. A. Einhellig contributes a review of the mode of action of phenolic acids. Many observed physiological effects (e.g., on ion uptake and water balance) are related to the initial effects of phenolics on membranes. Also considered are the often overlooked interactive effects of environmental and allelochemical stress.

The chapters on allelochemical modes of action cover a wide range of approaches. J. G. Romagni et al. present evidence showing that the lichen substance (–)-usnic acid is more inhibitory than the herbicide sulcotrione against *p*-hydroxyphenyl pyruvate dioxygenase, the enzyme responsible for plastoquinone biosynthesis. M. Wink reviews work done by Wink and colleagues on the physiology, synthesis, and mode of action of quinolizidine alkaloids, which occur in many legumes. Significant toxicity has been found with inhibition of Na^+ and K^+ ion channels, interactions with the nicotinic and muscarinic acetylcholine receptor and inhibition of protein synthesis. A chapter by M. S. Blum on the chemical ecology of alkaloids gives a good, general overview of their role in plant–insect and insect–insect relationships, but little on their possible role in plant–plant interactions. S. O. Duke and A. Oliva evaluate the literature on mode of action of terpenoids. Their excellent review will provide guidance for those seeking methods to investigate allelochemical modes of action. Sánchez-Moreiras et al. cover the mode of action of the benzoxazolinones, particularly effects seen on mitosis. H. G. Cutler et al. review the mode of action of a number of several phytotoxic fungal metabolites, comparing their mode of action in plants and in therapeutic systems. The possible role of toxins from phytopathogenic fungi are not often considered in most investigations of allelopathy, despite the fact that many have quite high activity. Chaetoglobosin K, for example, shows activity in wheat coleoptile assays down to 10^{-7} M. The application of proteomic techniques to study the effect of allelochemical stress is discussed by R. Cruz-Ortega et al. Clear changes in protein profiles can be observed by 2-dimensional gel electrophoresis. This technique seems to hold much promise. G. Aliotta et al. have taken a unique approach to allelopathy, investigating the potential utility of olive mill waste water for natural weed control. They discuss the application of microscopic techniques to gain insight into the mode of action of the waste water and specific compounds found in it. Results show that careful microscopy can provide invaluable clues about a compound's mode of action. Finally, a chapter by R. E. Hoagland and R. D. Williams covers bioassays. While glossing over some of the difficulties posed in establishing a laboratory assay that has meaning for the field situation, and overlooking some important cautions such as the effect of seed number and plant density on toxicity,

this is a fairly thorough review. Especially useful is the emphasis on the potential for bioassays to provide insight into allelochemical modes of action.

In summary, this volume will be a helpful resource on the chemistry of allelopathic interactions for both established researchers and graduate students beginning their research. Particularly for those contemplating research on allelochemical modes of action, this text is a must.

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INHIBITION OF MOUTH SKELETAL MUSCLE RELAXATION BY FLAVONOIDS OF *Cistus ladanifer* L.: A PLANT DEFENSE MECHANISM AGAINST HERBIVORES

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Abstract—*Cistus ladanifer* exudate is a potent inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase (Ca^{2+}-pump) of rabbit skeletal muscle, a well-established model for active transport that plays a leading role in skeletal muscle relaxation. The low concentration of exudate needed to produce 50% of the maximum inhibition of the sarcoplasmic reticulum Ca^{2+}-ATPase activity, 40–60 μg/ml, suggests that eating only a few milligrams of *C. ladanifer* leaves can impair the relaxation of the mouth skeletal muscle of herbivores, as the exudate reaches up to 140 mg/g of dry leaves in summer season. The flavonoid fraction of the exudate accounts fully for the functional impairment of the sarcoplasmic reticulum produced by the exudate (up to a dose of 250–300 μg/ml). The flavonoids present in this exudate impair the skeletal muscle sarcoplasmic reticulum function at two different levels: (i) by inhibition of the Ca^{2+}-ATPase activity, and (ii) by decreasing the steady state ATP-dependent Ca^{2+}-accumulation. Among the exudate flavonoids, apigenin and 3,7-di-*O*-methyl kaempferol are the most potent inhibitors of the skeletal muscle sarcoplasmic reticulum. We conclude that the flavonoids of this exudate can elicit an avoidance reaction of the herbivores eating *C. ladanifer* leaves through impairment of mouth skeletal muscle relaxation.

Key Words—Flavonoids, *Cistus ladanifer*, Ca^{2+}-ATPase, skeletal muscle, plant defense, apigenin, 3,7-di-*O*-methyl kaempferol.

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INTRODUCTION

Secondary metabolites production, accumulation, and release through evolution play a major role in plant survival under stressful conditions (Seigler, 1998; Chaves and Escudero, 1999). The importance of plant chemistry in the evolution of plant–herbivore interactions has been well documented. Among plant secondary metabolites, phenolic compounds have received particular attention because (1) they are present in many plant secretions (Wollenweber and Dietz, 1981; Seigler, 1998), (2) they are enriched in the parts of plants' most vulnerable areas to attack from other organisms (such as leaves and photosynthetic stems), and (3) they show variation in their chemical structure great enough to account for the variety of putative environmental insults from other living organisms.

Arinafril and Suwandi (2001) have pointed out that the presence of compounds in leaves and photosynthetic stems with bioactivity against herbivores eating can also account for plant protection, by acting as dissuasive compounds. Although plant phenolics are believed to play an important role in chemical defense, their specific physiological effects on herbivores are variable and poorly understood (Appel, 1993). It has been reported that they can behave as antifeedants (Zamora et al., 1999), digestibility reducers (Harborne, 1994), and toxins (Rosenthal and Berenbaum, 1991).

Eating plant leaves or tender stems by herbivores involves a significant mouth skeletal muscle exercise, and skeletal muscle sarcoplasmic reticulum plays a central role in the control of contraction/relaxation of the skeletal muscle (Entman and Van Winkle, 1986; Fleischer and Inui, 1989). The lipophilic properties of flavonoids point out that they reach the intracellular space by simple diffusion across the lipid bilayer of the plasma membrane. Therefore, they may reach the skeletal muscle fibers in herbivore mouth by diffusion, without need of previous absorption along the digestive track. Thus, they avoid the herbivore detoxification systems for xenobiotics in the liver (Coulson et al., 1984; Ortiz de Montellano, 1986).

The sarcoplasmic reticulum forms an extensive membrane network that surrounds the skeletal muscle myofibrils. Ca^{2+}-ATPase is by far the major protein component (Andersen, 1989). Ca^{2+}-ATPase couples the hydrolysis of ATP to active Ca^{2+}-transport into the sarcoplasmic reticulum lumen and is responsible for the rise of a high Ca^{2+}-concentration gradients ($\geq$10000) across this membrane in the relaxed state of the skeletal muscle cell (de Meis and Vianna, 1979). This protein plays a leading role in skeletal muscle relaxation in vertebrates, as inhibition of this Ca^{2+}-ATPase or a decrease in the capacity of the sarcoplasmic reticulum membrane to maintain a large Ca^{2+}-gradient results in an impaired relaxation of the skeletal muscle, and eventually leads to tetanic-like myofibril contractions when the cytosolic Ca^{2+} concentration increases to the micromolar range (Ruegg, 1988).

Shoshan and MacLennan (1981) reported that micromolar concentrations of the flavonoid quercetin strongly inhibit the sarcoplasmic reticulum Ca^{2+}-ATPase from rabbit skeletal muscle, and this Ca^{2+}-ATPase has been shown to be a good prototype for other herbivore and insect skeletal muscle Ca^{2+}-ATPases (Martonosi, 1984). Because of the need for proper function of skeletal muscle for eating by herbivorous organisms, leaves or photosynthetic stems containing high flavonoid concentrations may result in impaired mouth muscle relaxation and produce an avoidance reaction.

In previous studies, we have shown the high concentration of flavonoids in the exudate of *Cistus ladanifer* L. and their possible function as allelopathic agents (Chaves et al., 1993, 1998, 2001; Chaves and Escudero, 1999). The exudate is enriched in flavonoids during summer, particularly in flavonols (kaempferols) over flavones (apigenins) (Chaves et al., 1993, 1997). In the summertime, *C. ladanifer* is often exposed simultaneously to different types of stress (high levels of UV, drought, and high temperatures), and protection of the growing plant from herbivory should play a critical role in plant survival.

Only low doses of the *C. ladanifer* exudate are needed to impair the skeletal muscle sarcoplasmic reticulum capacity to sequester Ca^{2+}, and this effect can be accounted for by the flavonoids present in the exudate.

METHODS AND MATERIALS

Extraction, Purification, and Quantification of Flavonoids. C. ladanifer exudate was prepared as described in detail in a previous paper (Chaves et al., 1997). Apigenin was obtained from Aldrich Chemical (Madrid, Spain). Methylated flavonoids (3-*O*-methylkaempferol, 3,4′-di-*O*-methylkaempferol, 3,7-di-*O*-methylkaempferol, 4′-*O*-methylapigenin, and 7-*O*-methylapigenin) were prepared by HPLC from the *C. ladanifer* exudate as indicated in Chaves et al. (1998). Briefly, the exudate was dissolved in hot methanol and chilled at -20°C for 12 hr. Precipitated waxes were removed by centrifugation at 4°C (4500 $\times g$ for 10 min). The extract was loaded onto a 25 $\times$ 1.5 cm hydrated Sephadex LH-20 column (equilibrated with methanol for 24 hr), and eluted with methanol. Three fractions were obtained and subsequently analyzed by HPLC to determine which fraction contained the flavonoids. Flavonoids were separated on a semipreparative Nucleosil 5μ C-18 (250 $\times$ 10 mm) column using water–methanol–acetonitrile–tetrahydrofuran (56:16:6:22) as elution solvent. The flow rate was maintained at 1.75 ml/min. The flavonoids were detected with a diode array detector (350 nm). As each flavonoid was detected, it was collected into a separate tube. To eliminate any possible contamination from other compounds eluting close to the flavonoid of interest, the fraction was reseparated by HPLC with a methanol:water (80:20) at a flow rate of 2.5 ml/min.

TABLE 1. CONTENT OF *Cistus ladanifer* LEAF EXUDATE FLAVONOIDS

	Spring		Summer	
	Exudate (mg/g)	Dry mass (mg/g)	Exudate (mg/g)	Dry mass (mg/g)
Ap	1.43	0.17	1.68	0.23
K-3	8.58	0.99	11.80	2.31
Ap-4′	19.17	2.18	13.15	1.76
Ap-7	22.51	2.54	26.61	3.63
K-3,4′	17.35	1.97	33.71	4.47
K-3,7	32.34	5.95	161.96	21.98

Note. Ap: apigenin; K-3: 3-*O*-methylkaempferol; Ap-4′: 4′-*O*-methylapigenin; Ap-7: 7-*O*-methylapigenin; K-3,4′: 3,4′-di-*O*-methylkaempferol; K-3,7: 3,7-di-*O*-methylkaempferol. $N = 12$.

Quantitative analysis of leaf flavonoids was performed as indicated in previous publications (Chaves et al., 1993, 1997). Leaves (2–3 g; $N = 12$) were collected in spring and summer. They were dipped several times into chloroform (2 ml), and the chloroform was removed by evaporation. The exudate was dissolved in methanol (2 ml), and analyzed by HPLC as follows: extract (20 μl) was injected onto a Nucleosil 5μ C-18 (150 $\times$ 4 mm) column and eluted with water–methanol–acetonitrile–tetrahydrofuran (56:16:6:22) at a flow rate of 0.7 ml/min (Chaves et al., 1993, 1997). After extraction, leaves were weighed, oven-dried at 60°C for 12 hr, and reweighed again to determine dry biomass. The content of exudate flavonoids is indicated in the Table 1.

Preparation of Sarcoplasmic Reticulum Vesicles. Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle as indicated in Cuenda et al. (1994). Protein concentrations were measured following the method of Lowry et al. (1951), using bovine serum albumin as a standard. The Ca^{2+}-ATPase accounted for 75–80% of the total protein in sarcoplasmic reticulum membranes, as determined by densitometry of Coomasie blue stained SDS/gels (7–10% acrylamide) using a BioRad Molecular Imager® FX.

Ca^{2+}-ATPase Activity. Ca^{2+}-ATPase activity was measured spectrophotometrically at 25°C using the coupled enzyme system pyruvate kinase/lactate dehydrogenase as in Cuenda et al. (1990, 1994). The following reaction mixture was used: 0.1 M TES/KOH (pH 7.45), 0.1 M KCl, 0.1 mM $CaCl_2$, 3 mM $MgCl_2$, 2.5 mM ATP, 0.42 mM phosphoenolpyruvate, 0.25 mM NADH, 7.5 IU pyruvate kinase, and 18 IU lactate dehydrogenase. On average, sarcoplasmic reticulum membranes had less than 5% of Ca^{2+}-independent ATPase activity, measured in the presence of 5 mM EGTA. The specific Ca^{2+}-ATPase activity of sarcoplasmic reticulum membrane preparations was stimulated between 8 and 10-fold following addition of 0.04 μg of the Ca^{2+}-ionophore calcimycin per microgram of sarcoplasmic reticulum protein.

Ca^{2+} Accumulation by Sarcoplasmic Reticulum Vesicles. Steady state ATP-dependent Ca^{2+} accumulation by sarcoplasmic reticulum was measured at 25°C using arsenazo III as the metallochromic indicator from the difference of absorbance between 650 and 700 nm, as in Fernandez-Salguero et al. (1990). The following assay mixture was used: 0.1 M TES/KOH (pH 7.45), 0.1 M KCl, 0.1 mM $CaCl_2$, 3 mM $MgCl_2$, 2.5 mM ATP, and 100 μM arsenazo III. The conversion of absorbance change into Ca^{2+} concentration was carried out by calibration with EGTA Ca^{2+} solutions.

For titration studies, flavonoids were dissolved in DMSO at a concentration at least 100-fold the maximum concentration to be used in activity measurements. Control experiments confirmed that up to 1% DMSO did not produce any significant effect on the sarcoplasmic reticulum Ca^{2+}-ATPase activity nor on steady state ATP-dependent Ca^{2+} accumulation by sarcoplasmic reticulum vesicles.

Fluorescence. Steady-state fluorescence measurements were performed in a continuously stirred cuvette with the following spectrofluorimeters: Hitachi-Perkin–Elmer (model 650-40) and SLM-4800C, with 280 and 335 nm as excitation and emission wavelengths, respectively. Inner filter effects, due to the absorbance of flavonoids at 280 and 335 nm, were corrected using the following equation: $F_{corr} = C \times F_{obs}$, where F_{corr} and F_{obs} are the values of the corrected and observed fluorescence, and $C = \text{antilog}((A_{280} + A_{335})/2)$ (Lackowicz, 1983). Flavonoid extinction coefficients at 280 nm (ε_{280}) and 335 nm (ε_{335}) were determined in 0.1 M TES/0.1 M KCl and 3 mM $MgCl_2$ (pH 7), the buffer used for fluorescence measurements. The sum of the absorbance at 280 and 335 nm at the highest flavonoid concentrations used in the titration of intrinsic sarcoplasmic reticulum fluorescence (25–30 μM) was close to 0.3, and only for concentrations of the flavonoid higher than 10 μM was the value of the correction factor C higher than 1.2.

Egg lecithin liposomes containing 10% octyl-tryptophan were prepared by the ethanol injection method of Fung and Stryer (1978), and characterized as in a Antollini et al. (1996).

The experimental data obtained for inhibition of Ca^{2+}-ATPase activity and for fluorescence quenching by the flavonoids were analyzed by nonlinear least-squares two-parameters fit to the equations indicated in the text using the program Origin 5.0™.

RESULTS

Sarcoplasmic reticulum Ca^{2+}-ATPase was inhibited by the exudate of *C. ladanifer*. The summer exudate was several-fold more potent than the spring exudate (Figure 1A). Since the content of leaf flavonoids increased several-fold from spring to summer (Table 1 and Chaves et al., 1993, 1997), this suggested that *C. ladanifer* flavonoids are inhibitors of the Ca^{2+}-ATPase. This was confirmed using the flavonoid fraction, which showed that only 13 μg flavonoids/ml are needed to produce 50% inhibition of the sarcoplasmic reticulum Ca^{2+}-ATPase

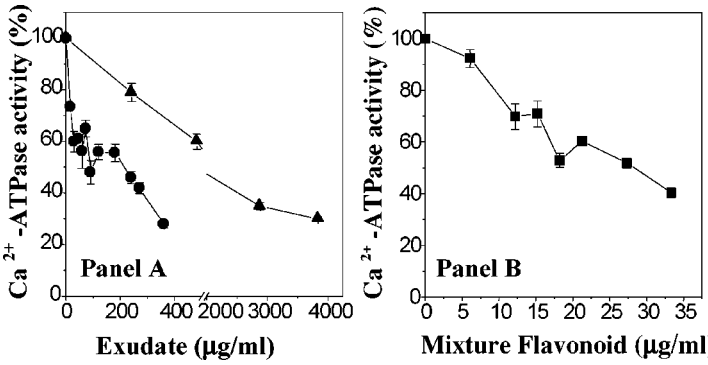

FIG. 1. Inhibition of the sarcoplasmic reticulum Ca^{2+}-ATPase activity by the spring (triangles) and summer (circles) *C. ladanifer* exudate (Panel A) and by the flavonoid mixture of the summer exudate (Panel B). Ca^{2+}-ATPase activity was assayed in the presence of 4% calcimycin.

(Figure 1B). Taking into account the molecular weight of the flavonoids present in the flavonoid fraction (Table 1 and Chaves et al., 1993, 1997), this implies that one or several of the components inhibit the sarcoplasmic reticulum Ca^{2+}-ATPase with an inhibitory dissociation constant in the micromolar range.

Inhibition of the Ca^{2+}-ATPase activity is not due to solubilization of the sarcoplasmic reticulum membranes by the exudate nor by the flavonoid fraction, because the light scattering of the membranes decreases less than 10% in the presence of exudate and flavonoid fractions producing more than 50% inhibition of the Ca^{2+}-ATPase activity (data not shown). Furthermore, in the absence of the Ca^{2+} ionophore calcimycin, the Ca^{2+}-ATPase activity is largely inhibited in well-sealed sarcoplasmic reticulum vesicles by the Ca^{2+} gradient established by the operation of the Ca^{2+}-pump (de Meis and Vianna, 1979; Entman and Van Winkle, 1986; Andersen, 1989). This is shown experimentally by a large stimulation of the Ca^{2+}-ATPase by the Ca^{2+} ionophore, calcimycin (Table 2). Table 2 also shows that, in presence of exudate and flavonoid fraction concentrations that produced a large inhibition of the Ca^{2+}-ATPase activity, the ratio between Ca^{2+}-ATPase activity in the absence and presence of calcimycin is much lower than 1, and closer to that found for control (well sealed) sarcoplasmic vesicles. Therefore, inhibition of Ca^{2+}-ATPase activity cannot be rationalized in terms of a large perturbation of the sarcoplasmic reticulum membrane structure, which should be monitored by a loss of the large Ca^{2+} gradient generated across this membrane by Ca^{2+}-ATPase activity. In fact, impairment of the sarcoplasmic reticulum vesicles to accumulate Ca^{2+} required concentrations of the summer exudate (LD_{50} about 500 μg/ml, Table 3) much higher than the IC_{50} value obtained for the inhibition of Ca^{2+}-ATPase activity.

TABLE 2. EFFECTS OF *Cistus ladanifer* SUMMER EXUDATE AND THE EXUDATE FLAVONOID FRACTION ON CA^{2+}-GRADIENT INHIBITION OF THE SARCOPLASMIC RETICULUM CA^{2+}-ATPase

Sarcoplasmic reticulum	Ca^{2+}-ATPase activity ratio (−calcimycin/+calcimycin)
Control	0.10 ± 0.04
+30 μg exudate/ml	0.17 ± 0.04
+120 μg exudate/ml	0.32 ± 0.04
+15 μg flavonoids/ml	0.14 ± 0.04
+33 μg flavonoids/ml	0.25 ± 0.04

Note. $N = 5$.

Flavonoids are lipophilic compounds, and should be expected to strongly adsorb to biological membranes, such as the sarcoplasmic reticulum membrane, thereby promoting specific functional perturbations that lead to altered physiological responses. Thus, we have measured the adsorption of the flavonoids used in this work onto the sarcoplasmic reticulum membranes.

As flavonoids quench the intrinsic fluorescence of the sarcoplasmic reticulum membranes (Figure 2), and more than 80% of the intrinsic fluorescence of sarcoplasmic reticulum (largely Trp fluorescence) is accessible to quenching through the lipid bilayer (London and Feigenson, 1981), we made use of this property to monitor the incorporation of flavonoids into sarcoplasmic reticulum membranes

TABLE 3. EFFECTS OF *Cistus ladanifer* SUMMER EXUDATE ON STEADY STATE ATP-DEPENDENT CA^{2+} ACCUMULATION BY THE SARCOPLASMIC RETICULUM

Exudate (μg/ml)	Ca^{2+} uptake (% of control)[a]
0 (control)	100
30	98
180	100
360	85.5
450	74.6
540	35
720	28
2160	30

Note. $N = 3$.

[a] The control value of measured steady state ATP-dependent Ca^{2+} uptake was 99.3 nmoles Ca^{2+}/mg sarcoplasmic reticulum protein.

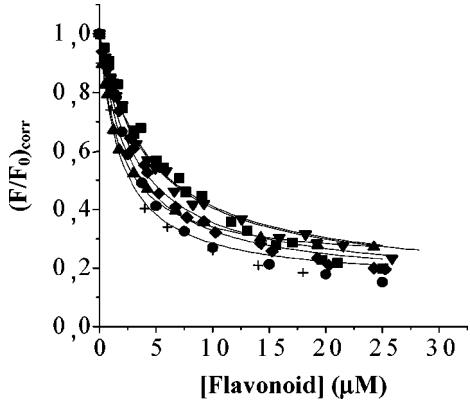

FIG. 2. Quenching of intrinsic fluorescence of sarcoplasmic reticulum membranes by *C. ladanifer* exudate flavonoids. Symbols correspond to apigenin (squares), 4′-*O*-methylapigenin (up-triangles), 7-*O*-methylapigenin (down-triangles), 3-*O*-methylkaempferol (diamonds), 3,4′-di-*O*-methylkaempferol (+), and 3,7-di-*O*-methylkaempferol (circles). The lines are the best nonlinear least squares fit of the data to the equation indicated in the text, which yielded the following IC_{50} values: 5 μM ($\chi^2 = 0.0011$) for apigenin; 3.2 μM ($\chi^2 = 0.0034$) for 4′-*O*-methylapigenin; 1.8 μM ($\chi^2 = 0.0001$) for 7−*O*−methylapigenin; 4.5 μM ($\chi^2 = 0.0003$) for 3-*O*-methylkaempferol; 3.6 μM ($\chi^2 = 0.0011$) for 3,4′-di-*O*-methylkaempferol; 1.9 μM ($\chi^2 = 0.0012$) for 3,7-di-*O*-methylkaempferol.

by using a methodological approach similar to that outlined in Gutiérrez-Merino et al. (1989). Briefly, we measured: (1) the intrinsic fluorescence of sarcoplasmic reticulum vesicles (Figure 2), and (2) the fluorescence of Trp incorporated as *n*-octyl Trp into egg lecithin liposomes (Figure 3) in the presence of different concentrations (up to 50 μM) of each flavonoid in a pH 7 buffered solution. The data for each flavonoid were fitted (using the iterative nonlinear squares two parameters fit algorithm of Origin 5.0^{TM} software) to the following equation:

$$\frac{F}{F_0} = 1 - \left\{ \frac{F_{\min}(\text{Flavonoid})}{IC_{50} + [\text{Flavonoid}]} \right\},$$

where F_0 and $F_{\min}$ are the fluorescence intensity in the absence and at saturation of the flavonoid, respectively; IC_{50} is the concentration of flavonoid that produces 50% of the maximum fluorescence quenching attained at saturation by the flavonoid, and F is the fluorescence in the presence of each concentration of flavonoid. The two parameters that were fit by iteration were $F_{\min}$ and IC_{50}. The flavonoids studied are more potent as quenchers of Trp fluorescence in a lipid phase than in an aqueous environment (compare Figures 3 and 4), reflecting the large partition of these flavonoids into the lipid bilayer. To obtain the IC_{50} value for the

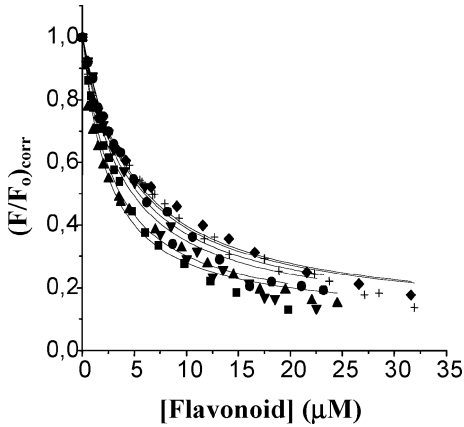

FIG. 3. Quenching of octyl-Trp incorporated in to egg lecithin liposomes by *C. ladanifer* exudate flavonoids. Octyl-Trp was incorporated into egg lecithin liposomes at a molar ratio of 1% octyl-Trp/lipid. Different symbols correspond to the different flavonoids present in the exudate (as in Figure 2). The lines are the best nonlinear least squares fit of the data to the equation indicated in the text, which yielded the following IC_{50} values: 3 μM ($\chi^2 =$ 0.0013) for apigenin; 4.5 μM ($\chi^2 = 0.0017$) for 4′-*O*-methylapigenin; 2.7 μM ($\chi^2 =$ 0.001) for 7-*O*-methylapigenin; 4.2 μM ($\chi^2 = 0.0033$) for 3-*O*-methylkaempferol; 5.3 μM ($\chi^2 = 0.001$) for 3.4′-di-*O*-methylkaempferol; 5.1 μM ($\chi^2 = 0.001$) for 3.7-di-*O*-methylkaempferol.

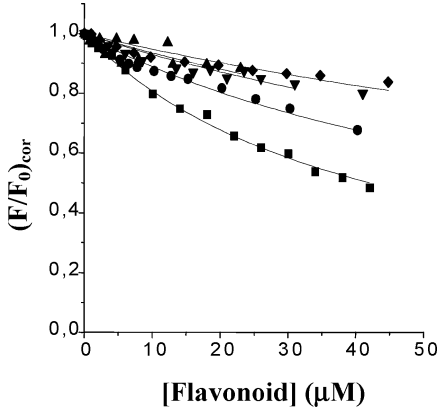

FIG. 4. Quenching of Trp fluorescence in aqueous solution by flavonoids in *C. ladanifer* exudate. The symbols correspond to apigenin (squares); 4′-*O*-methylapigenin (circles); 7-*O*-methylapigenin (up-triangles); 3-*O*-methylkaempferol (down-triangles); and 3,4′-di-*O*-methylkaempferol (diamonds).

sarcoplasmic reticulum by iteration, F_{min}/F_0 was allowed to vary between 0.15 and 0.25, taking into account previous studies on the quenching of intrinsic sarcoplasmic reticulum fluorescence by lipid quenchers (London and Feigenson, 1981).

The lipid concentrations used in the titration of Trp fluorescence shown in the Figures 2 and 3 were similar (the molar lipid/Ca^{2+}-ATPase ratio in sarcoplasmic reticulum membranes is 90 ± 10 (Entman and Van Winkle, 1986). Therefore, the ratio between the IC_{50} values obtained for egg lecithin liposomes with 10% octyl-tryptophan, $(IC_{50})_{PC}$, and for sarcoplasmic reticulum vesicles, $(IC_{50})_{SR}$, is proportional to the partition coefficient of the flavonoid in the sarcoplasmic reticulum membrane relative to that into egg lecithin lipid bilayer, $K_{SR/PC}$.

The values obtained for $(IC_{50})_{PC}$, $(IC_{50})_{SR}$, and $K_{SR/PC}$ for each flavonoid are listed in Table 4. These flavonoids produced 50% quenching of the intrinsic fluorescence of sarcoplasmic reticulum vesicles at molar ratios ranging between 0.04 and 0.1 moles of flavonoid per mole of sarcoplasmic reticulum lipid. In addition, 3,7-di-*O*-methylkaempferol is adsorbed to the sarcoplasmic reticulum membrane more strongly than all of the other flavonoids, i.e., more than a twofold higher incorporation than in egg lecithin liposomes.

In summer, the season of the year in which the secretion of exudate by *C. ladanifer* reaches its maximum value, the predominant flavonoids are (percentage of total flavonoids given in parenthesis): 3,7-di-*O*-methylkaempferol (65.07%) > 3,4′-di-*O*-methylkaempferol (13.54%) > 7-*O*-methylapigenin (10.69%) > 4′-*O*-methylapigenin (5.28%) > 3-*O*-methylkaempferol (4.74%) > apigenin (0.67%) (Table 1; see also Chaves et al., 1993, 1997).

The compounds 3,7-di-*O*-methylkaempferol, 3,4′-di-*O*-methylkaempferol, 3-*O*-methylkaempferol, 7-*O*-methylapigenin, and apigenin inhibited Ca^{2+}-ATPase activity (Figure 5). Only 4′-*O*-methylapigenin (up to 100 μM) had less than 20% effect on sarcoplasmic reticulum Ca^{2+}-ATPase activity in the presence of calcimycin (to relieve it from inhibition by the Ca^{2+}-gradient across the sarcoplasmic reticulum membrane). Inhibition produced by these flavonoids is rapid,

TABLE 4. CONCENTRATIONS OF *Cistus ladanifer* EXUDATE FLAVONOIDS THAT PRODUCED HALF-QUENCHING OF INTRINSIC FLUORESCENCE OF SARCOPLASMIC RETICULUM MEMBRANES AND OF THE FLUORESCENCE OF OCTYL-TRYPTOPHAN IN EGG LECITHIN LIPOSOMES

Flavonoid	$(IC_{50})_{PC}$ (μM)	$(IC_{50})_{SR}$ (μM)	$K_{SR/PC}$
Apigenin	3.0 ± 0.3	5.0 ± 0.5	0.6 ± 0.15
4′-*O*-Methylapigenin	4.5 ± 0.4	3.2 ± 0.3	1.4 ± 0.25
7-*O*-Methylapigenin	2.7 ± 0.3	1.8 ± 0.2	1.5 ± 0.3
3-*O*-Methylkaempferol	4.2 ± 0.4	4.5 ± 0.5	0.9 ± 0.2
3,4′-Di-*O*-Methylkaempferol	5.3 ± 0.4	3.6 ± 0.4	1.5 ± 0.25
3,7-Di-*O*-Methylkaempferol	5.1 ± 0.4	1.9 ± 0.3	2.7 ± 0.5

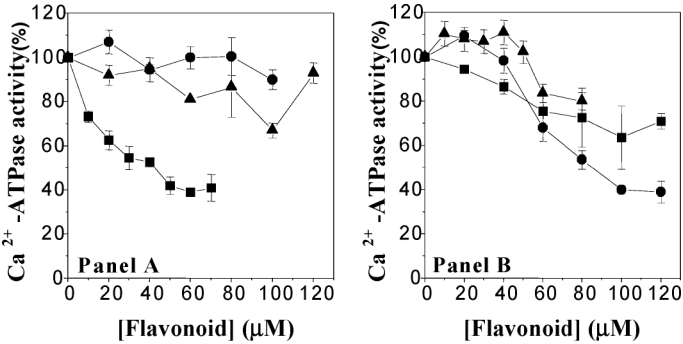

FIG. 5. Effects of different *C. ladanifer* flavonoids on the Ca^{2+}-ATPase activity of sarcoplasmic reticulum membranes in the presence of calcimycin. Panel A: Exudate apigenins: apigenin (squares); 4′-*O*-methylapigenin (circles); and 7-*O*-methylapigenin (up-triangles). Panel B: Exudate kaempferols: 3-*O*-methylkaempferol (squares); 3,4′-di-*O*-methylkaempferol (circles); and 3,7-di-*O*-methylkaempferol (up-triangles).

i.e., it develops in less than 30 sec after mixing of the solutions and remains constant for at least 1 hr. The maximum inhibition attained for each flavonoid (I_{max}) and the concentration of the flavonoid that produced 50% of the maximum inhibition (IC_{50}) are listed in Table 5. However, for methylated kaempferols, these values should be interpreted as apparent IC_{50} values, because of the biphasic pattern shown by the plots of the Ca^{2+}-ATPase activity versus the concentration of these flavonoids in the presence of calcimycin (apparent lag phase at the lower flavonoid concentrations tested, Figure 5).

TABLE 5. KINETIC PARAMETERS OF THE MODULATION OF THE CA^{2+}-ATPase ACTIVITY OF SARCOPLASMIC RETICULUM MEMBRANES BY THE *Cistus ladanifer* EXUDATE FLAVONOIDS

	−Calcimycin		+Calcimycin	
Flavonoid	Stimulation	IC_{50} (μM)	I_{max} (%)	IC_{50} (μM)
Apigenin	−	—	65 ± 5	12 ± 2
4′-*O*-Methylapigenin	+	> 100	< 20	—
7-*O*-Methylapigenin	+	70–80	30 ± 5	40 ± 5
3-*O*-Methylkaempferol	−	—	35 ± 5	45 ± 5
3,4′-Di-*O*-Methylkaempferol	−	—	65 ± 5	55 ± 5
3,7-Di-*O*-Methylkaempferol	+ + +	25–30	30 ± 5	60 ± 5

The Ca^{2+}-ATPase activity was measured in the absence/presence of 4% calcimycin (to relieve the inhibition by Ca^{2+} gradient across the sarcoplasmic reticulum membrane). $N = 5$.

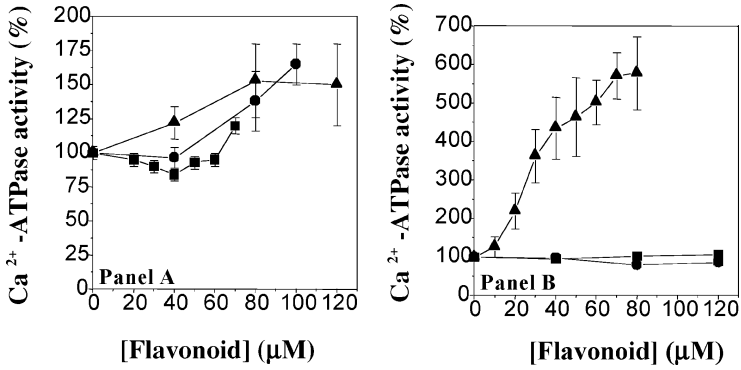

FIG. 6. Effects of the different *C. ladanifer* exudate flavonoids on Ca^{2+}-ATPase activity of sarcoplasmic reticulum membranes in the absence of calcimycin. Panel A: Exudate apigenins: apigenin (squares); 4′-*O*-methylapigenin (circles); and 7-*O*-methylapigenin (up-triangles). Panel B: Exudate kaempferols: 3-*O*-methylkaempferol (squares); 3,4′-di-*O*-methylkaempferol (circles); and 3,7-di-*O*-methylkaempferol (up-triangles).

In contrast, the flavonoids that are less potent as inhibitors of the Ca^{2+}-ATPase activity in presence of calcimycin—4′-*O*-methylapigenin, 7-*O*-methylapigenin, and 3,7-di-*O*-methylkaempferol—were found to stimulate Ca^{2+}-ATPase activity of sealed sarcoplasmic reticulum vesicles when assayed in the absence of calcimycin (Figure 6). Among these flavonoids, 3,7-di-*O*-methylkaempferol stimulated a higher Ca^{2+}-ATPase activity in the absence of calcimycin (Figure 6 and Table 5). All the other flavonoid (up to approximately 100 μM) produced less than 20% stimulation of the Ca^{2+}-ATPase activity in the absence of calcimycin, and no significant decrease (less than 10%) of the steady state ATP-dependent Ca^{2+} accumulation by sarcoplasmic reticulum vesicles. Stimulation of the Ca^{2+}-ATPase activity by methylated apigenins and 3,7-di-*O*-methylkaempferol correlated with a decrease in (close to 30%) steady state ATP-dependent Ca^{2+}-accumulation by sarcoplasmic reticulum vesicles. The flavonoid 3,7-di-*O*-methylkaempferol was the more potent of them, as 60 μM of this flavonoid produced the same effect as 100 μM of the methylated apigenins.

Most of the flavonoids of *C. ladanifer* strongly impair the ability of skeletal muscle sarcoplasmic reticulum to sequester Ca^{2+} either through inhibition of the Ca^{2+}-ATPase activity or through a decrease of the steady state ATP-dependent accumulation of Ca^{2+}.

DISCUSSION

C. ladanifer exudate impaired the ability to sequester and/or to accumulate Ca^{2+} by the skeletal muscle sarcoplasmic reticulum. The flavonoids within this

exudate strongly adsorb onto the sarcoplasmic reticulum membrane and can account for the functional perturbations of the sarcoplasmic reticulum produced at low summer leaf exudate doses (i.e., up to near 450 μg/ml). Because of the well-established leading role of Ca^{2+} uptake by the sarcoplasmic reticulum to promote skeletal muscle relaxation (Ruegg, 1988), this suggests that *C. ladanifer* exudate flavonoids are potent bioactive agents that impair skeletal muscle relaxation. The most potent flavonoid in the exudate is 3,7-di-*O*-methylkaempferol, the flavonoid that becomes most enriched in the *C. ladanifer* exudate from spring to summertime (Chaves et al., 1997). The decrease of the ATP-dependent steady state Ca^{2+} accumulation in sarcoplasmic reticulum, such as that produced by 3,7-di-*O*-methylkaempferol and to a minor extent by 4′-*O*-methyl and 7-*O*-methylapigenin, produces a typical phenotype of flaccid muscle paralysis associated with a long lasting rise of cytosolic Ca^{2+} (Ruegg, 1988).

Flavonoid impairment of sarcoplasmic reticulum function is a rapid effect. The results obtained with egg lecithin liposomes (Figure 3) showed that *C. ladanifer* exudate flavonoids strongly partition into the lipid bilayer, indicating that they can rapidly reach the intracellular spaces, and into exposed tissues by lipid-mediated diffusion. Owing to the anatomical disposition of mouth skeletal muscles in herbivores, these flavonoids could be expected to impair mastication of leaves above a threshold dose and induce an avoidance reaction of the herbivores for eating *C. ladanifer* leaves. Thus, within the conceptual framework of Arinafril and Suwandi (2001), *C. ladanifer* exudate flavonoids can be categorized as dissuasive compounds for herbivores. The exudate includes *C. ladanifer* leaves and photosynthetic stems and should afford an efficient protection against herbivores to the most vulnerable parts of the plant. An approximate threshold dose can be estimated from the overall volume of tissue exposed to flavonoids in the mouth of the herbivores (approximately 10 ml for an adult rabbit) and from the flavonoid composition of the exudate. Since *C. ladanifer* exudate shows a strong seasonal variation (Chaves et al., 1993, 1997; see also Table 1), the threshold dose should be expected to vary as well.

In summer, the exudate secretion reaches a peak of 140 mg/g of leaf dry weight (Chaves et al., 1997). Figure 1 suggests that only a few milligrams of *C. ladanifer* leaves (dry weight) are needed to produce an impaired relaxation of the mouth skeletal muscles of a rabbit ($>25\%$ functional decrease of the sarcoplasmic reticulum ability for Ca^{2+} withdrawal, either by inhibition of the Ca^{2+}-ATPase or by decrease of the ATP-dependent steady state accumulation of Ca^{2+}). For smaller herbivores, such as insects, the amount of *C. ladanifer* leaves needed will be lower, roughly proportional to their relative mouth sizes, as their skeletal muscle Ca^{2+}-ATPases show large functional and structural homology with rabbit skeletal muscle Ca^{2+}-ATPase (Entman and Van Winkle, 1986; Andersen, 1989). During the spring season, the amount of exudate per gram of dry leaves is only about one third of that in summertime (Chaves et al., 1993, 1997), with significant

quantitative variation in the flavonoid composition in the summer season (see Table 1). The potency of the summer exudate as an inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase was found to be between four to sixfold higher than the spring exudate (Figure 1A). This closely correlates with the fourfold increase of 3,7-di-*O*-methylkaempferol plus 3,4′-di-*O*-methylkaempferol content in the exudate. However, impairment of sarcoplasmic reticulum function by summer exudate should be longer lasting than that afforded by the spring exudate, due to the enrichment in 3,7-di-*O*-methylkaempferol (see above). Seasonal variation of exudate flavonoids is by far the most relevant ecological variable, because other variables, i.e., average rainfall and UV irradiation, produce at most a twofold change in the exudate secretion, with only minor quantitative variations of the flavonoid composition (<20% variation in the content of total apigenins and kaempferols) (Chaves et al., 1997).

Thus, only 0.5–1 fresh *C. ladanifer* leaf (e.g., 20–40 mg dry-weight) should be needed to produce a long-lasting functional impairment of the mouth skeletal muscle of insect herbivores, and should elicit an avoidance reaction.

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THE ROLE OF SEMIOCHEMICALS IN THE AVOIDANCE OF THE SEVEN-SPOT LADYBIRD, *Coccinella septempunctata*, BY THE APHID PARASITOID, *Aphidius ervi*

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Abstract—The role of semiochemicals in mediating intraguild interactions between the seven-spot ladybird, *Coccinella septempunctata*, and the aphid parasitoid, *Aphidius ervi*, was investigated. Female parasitoids avoided leaves visited by *C. septempunctata* adults and larvae during the previous 24 hr. Ethanol extracts of *C. septempunctata* adults and larvae also induced avoidance responses by *A. ervi*. Two of the hydrocarbons identified by gas chromatography (GC) and coupled GC-mass spectrometry (GC-MS), *n*-tricosane ($C_{23}H_{48}$), and *n*-pentacosane ($C_{25}H_{52}$), when tested individually at levels found in the adult extract, induced avoidance by *A. ervi*. Further investigation of the larvae extract, and footprint chemicals deposited by adults in glass Petri dishes, confirmed the presence of the hydrocarbons. Parasitism rates of the pea aphid, *Acyrthosiphon pisum*, on broad bean plants, *Vicia faba*, which had been sprayed with a mixture of the chemicals, were lower than those on control plants. The effect, however, was no longer evident if parasitoid foraging was delayed by 24 hr after the plants were treated. The ecological significance of intraguild avoidance behavior and implications for possible use of the semiochemicals involved in future biological control programs are discussed.

Key Words—Intraguild predation, predator avoidance, trail, oviposition decision, biological control, *n*-tricosane, *n*-pentacosane.

INTRODUCTION

Trophic interactions among organisms sharing the same resource, specifically those interactions involving intraguild predation (IGP), have been well documented in

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several studies (Rosenheim et al., 1993; Colfer and Rosenheim, 1995; Ferguson and Stiling, 1996; Rosenheim, 1998; Raymond et al., 2000; Snyder and Ives, 2001). These reports suggest that IGP changes the extent to which top-down forces by predator guilds affect herbivore populations. Although less is known about the avoidance of intraguild predation, it has been documented both in predator–predator (Doumbia et al., 1998) and predator–parasitoid (Taylor et al., 1998; Nakashima and Senoo, 2003) interactions. In intraguild predator avoidance, the species at risk (i.e., intraguild prey) tends to avoid patches or microhabitats where the aggressors (i.e., intraguild predators) are already or potentially present. Such avoidance behavior may be categorized into two types, avoidance by the potential victims themselves, or avoidance by the parents of victims (e.g., oviposition avoidance).

There are several reports of intraguild avoidance behavior in aphidophagous predators. For example, chrysopid and coccinellid females were deterred from ovipositing when exposed to areas where heterospecifics were present (Ruzicka, 1998, 2001; Agarwala et al., 2003). However, few studies of intraguild predator avoidance have been conducted on predator–parasitoid interactions. In this system, parasitoids are usually the intraguild prey because parasitized hosts are potentially consumed by predators (Wheeler et al., 1968; Hoelmer et al., 1994; Wells et al., 2001). The aphid parasitoid, *Aphidius ervi* Haliday (Hymenoptera: Braconidae) avoids places where the intraguild predator, the seven-spot ladybird, *Coccinella septempunctata* L. (Coleoptera: Coccinellidae) is present or was present recently (Taylor et al., 1998). Such avoidance behavior is rationalized by the detection of chemical trails left by the predator. No studies, however, have identified the semiochemicals mediating this interaction.

The pea aphid, *Acyrthosiphon pisum*, is attacked by a large guild of arthropod natural enemies (Wheeler, 1974, 1977), in which *A. ervi* and *C. septempunctata* are usually the dominant species (Ekbom, 1994; Takahashi, 1996; Senoo et al., 2002). The seasonal distribution of *A. ervi* overlaps with that of foraging *C. septempunctata* ladybirds on alfalfa (Takahashi, 1996), and parasitized aphids can be consumed by the ladybirds (Nakashima, unpublished data). Using *A. pisum*, *A. ervi*, and *C. septempunctata* as the model prey—predator–parasitoid system, the chemical characteristics of trails left by foraging ladybirds on plant surfaces, and effects of these chemical compounds on parasitoid behavior were investigated in a series of laboratory experiments. The objectives of our study were to (1) identify the semiochemicals involved in this intraguild predator avoidance, (2) determine the effective period over which the avoidance behavior functions, and (3) determine the effects of specific compounds on avoidance behavior and parasitism rates of aphids. The overall objective was to assess the role of semiochemicals involved in intraguild predator avoidance, and to consider their potential use in the enhancement of parasitoids in biological control programs.

METHODS AND MATERIALS

Insects. *Aphidius ervi* were obtained from a laboratory colony that had been initiated with mummies of pea aphids, *A. pisum*, collected from pea fields in Hertfordshire and Bedfordshire, UK during spring 2001. Overwintered adult seven-spot ladybirds, *C. septempunctata*, were collected from evergreen shrubs at Rothamsted Research during March and April, 2002. Both parasitoids and ladybirds were kept at 20°C and a 16L:8D photoperiod, and maintained with *A. pisum*, reared on broad bean plants, *Vicia faba* L. (Fabaceae) (var. Sutton), until further use. Parasitoids were removed from the colony as final instar larvae or pupae at the mummy stage, and were kept in Petri dishes (9-cm diam, 1.5-cm height) containing cotton wool with honey solution as adult food until emergence. Two days after the first adult emergence was observed, females were individually confined in small Petri dishes (5.0-cm diam, 1.5-cm height) with approximately 50 *A. pisum* and cotton with honey solution. All females used in bioassays were 4 to 5-days old.

Ladybird Chemicals. *Coccinella septempunctata* adults (500, mixed sex) and fourth-instar larvae (150) were cooled with liquid nitrogen and extracted with freshly distilled ethanol (400 and 50 ml, respectively) for 24 hr at 25°C. The extracts and washings were recovered by filtration (gravity), and diluted to a volume of 500 and 150 ml, respectively, i.e., 1 ladybird equivalent/ml ethanol. A portion of the adult extract (100 ml) was subjected to distillation under high vacuum (0.04 Torr) for 24 hr at 25°C, as described by Pickett and Griffiths (1980), to produce a distillate (100 ml) containing volatile components, and a residue, containing components with little or no volatility, that was redissolved in ethanol (100 ml). The crude extract, along with the vacuum distillate and residue fractions, was kept at 4°C until use in the bioassays.

Components with little or no volatility in the adult extract were separated by repeating the vacuum distillation on another portion of the extract (100 ml), and subjecting the residue to liquid chromatography over Florisil (60–100 mesh, Aldrich Chemical Company, Gillingham, UK) using distilled hexane, diethyl ether, and ethanol as eluants (150 ml each). The fractions were evaporated to dryness and redissolved in ethanol (100 ml) prior to use in bioassays. For GC/GC-MS analysis, a portion of the hexane eluant that had been dissolved in ethanol was retained (50 ml) and evaporated to dryness to yield a waxy solid (10.00 mg), from which a solution in hexane (1 mg/ml) was prepared. The larval extract (50 ml) was also separated into hexane, diethyl ether, and ethanol eluting fractions by Florisil column chromatography. For GC/GC-MS analysis, a portion of the hexane eluant (25 ml) was retained and evaporated to dryness to yield a waxy solid (3.12 mg), from which a solution in hexane (1 mg/ml) was prepared. Compounds in the hexane solutions were quantified on the basis of percentage of the total integrated peak area. Male and female adult footprint extracts were collected by placing ladybirds in clean Petri dishes (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). Extracts were evaporated to 10 μl under a gentle stream of high purity nitrogen and stored in microvials at -20°C until further use.

Gas Chromatography (GC). Hexane eluants arising from liquid chromatography of the adult and larval ladybird extracts, and the adult footprint extracts, prepared as described above, were analyzed on an Hewlett-Packard 5880A gas chromatograph equipped with a split–splitless injector, a flame-ionization detector (FID), and a 10 m $\times$ 0.53 mm i.d. HP-1 bonded-phase fused-silica capillary column. The oven temperature was maintained at 40°C for 1 min, then programmed at 10°C/min to 150°C, held at this temperature for 0.1 min, then programmed at 10°C/min to 250°C. The carrier gas was hydrogen.

Coupled GC-Mass Spectrometry (GC-MS). A capillary GC column (50 m $\times$ 0.32 mm i.d. HP-1) fitted with a cool on-column injector was directly coupled to a mass spectrometer (VG Autospec, Fisons instruments, UK). Ionization was by electron impact at 70 eV, 250°C. The oven temperature was maintained at 30°C for 5 min, and then programmed at 5°C/min to 250°C. The carrier gas was helium. Tentative identifications by GC-MS were confirmed by peak enhancement on GC with authentic samples obtained from commercial sources (Pickett, 1990).

Chemicals. *n*-Tricosane ($C_{23}H_{48}$), *n*-pentacosane ($C_{25}H_{52}$), and *n*-heptacosane ($C_{27}H_{56}$) (all 99% purity) were purchased from the Aldrich Chemical Company (Gillingham, UK). For behavioral studies, individual and mixed solutions of these chemicals were prepared in distilled ethanol at the concentrations at which they were found in the ladybird extracts. All solvents used were distilled prior to use.

Application of Chemicals on Plants. Ladybird extracts and pure authentic compounds in ethanol were applied on broad bean seedlings (6–8 leaf stage, 1 plant per pot) in plastic plant pots (8.0 cm in height and 8.0 cm in diameter) for use in behavioral bioassays. The solutions described above were applied to the plants 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. A small peristaltic pump was fitted to the atomizer to provide stable control of flow rate. 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/m^2. Control leaves were prepared in a similar manner using distilled ethanol only.

Leaf Square Experiments. The effects of *C. septempunctata* trails and extracts on *A. ervi* responses were investigated by using a dual-choice bioassay. Treatment leaves containing trails were prepared by inserting a leaf from a bean plant (6–8 leaf stage) into a plastic container via a slit, releasing a ladybird adult or larva 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. Treated and control leaves containing extracts/pure compounds or solvents, respectively, were

prepared by using a sprayer as described above. Leaves were used for experiments either immediately (0 hr) or 24 hr 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 *A. ervi* female was then released. After allowing the parasitoid to settle (1 min), the time spent on each leaf square was measured for a period of 10 min. Each experiment was repeated × 20.

Effects of Ladybird Chemicals on Aphid Parasitism. Treated plants were sprayed with a mixed solution of *n*-tricosane and *n*-pentacosane in ethanol at the concentrations found in the ladybird extracts, and control plants were sprayed with distilled ethanol. Fifty mixed age *A. pisum* were released onto single treated and control bean plants, placed individually in cylindrical cages (30-cm diam × 30-cm height). A single *A. ervi* female was released into each cage and removed after 18 hr. Parasitism rates of *A. pisum* were estimated by rearing the aphids on the tested plants in the laboratory, each plant being kept in a cylindrical tube (10-cm diam × 24.5-cm height) for 10–14 days in a growth room (20°C, photoperiod of 16L:8D) to allow mummy formation. Rates of parasitism were estimated by dividing the number of mummified aphids by the initial number of released aphids. Experiments were initiated both 0 and 24 hr after spraying. Each experiment was repeated ×20.

Statistical Analysis. The durations of visits by *A. ervi* to treated and control leaf squares were analyzed with Wilcoxon's signed rank tests. Rates of parasitism were also compared by Wilcoxon's signed rank tests. Additive effects of chemical compounds on the degree of avoidance were analyzed by a two-way ANOVA with application of each chemical compound as main effects. The data were transformed to logarithms to stabilize the variance before this analysis.

RESULTS

In dual-choice-leaf-square bioassays conducted immediately after leaf exposure to *C. septempunctata* adults and larvae, the total residence times of *A. ervi* females on treated leaf squares were significantly shorter than on control leaves. This effect was not observed when leaves were tested 24 hr after exposure to the ladybird (Figure 1a). Similar results were also obtained for leaf squares sprayed with crude ethanol extracts of *C. septempunctata* adults and larvae (Figure 1b). However, when the crude adult extract was separated by vacuum distillation into a distillate containing volatile components and a residue containing components with little or no volatility, *A. ervi* responses varied. The residence time on leaf squares treated with the residue was significantly shorter than on control squares, and statistically similar to that for the crude extract, whereas the distillate showed little biological activity (Figure 2). Further separation of the

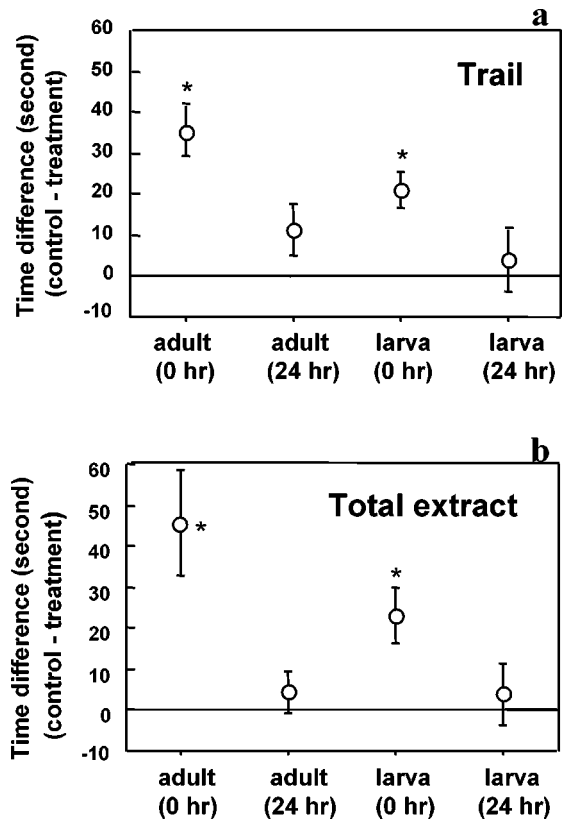

FIG. 1. Effect of seven-spot ladybird, *Coccinella septempunctata*, trails and extracts on residence times of *Aphidius ervi* females on broad bean leaf squares tested immediately or 24 hr after treatment. (a) *Coccinella septempunctata* adult and larval trails and (b) ethanol extracts of whole adult and larval *C. septempunctata*. Data are expressed as differences from residence times on control leaf squares in choice bioassays (control time—treatment time). Vertical lines with plots indicate ± SE. Asterisks indicate significant difference from control (Wilcoxon's signed rank test, $P < 0.05$).

residue by liquid chromatography over Florisil using high purity hexane, diethyl ether, and ethanol as eluants yielded three fractions comprising compounds of increasing polarity. Of these, the hexane-eluted fraction retained biological activity, suggesting that nonpolar chemicals were responsible for the parasitoid avoidance behavior. The diethyl ether and ethanol-eluted fractions showed little or no activity (Figure 3).

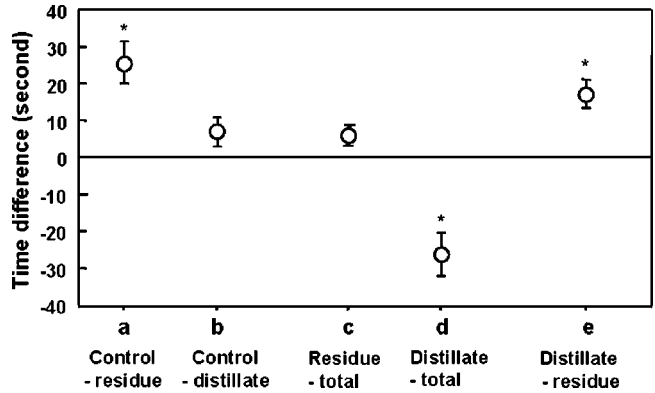

FIG. 2. Effect of vacuum distillation fractions of adult *Coccinella septempunctata* ethanol extract on residence times of *Aphidius ervi* females on broad bean leaf squares. Data are expressed as differences from residence times on control leaf squares (a, b), differences from residences times on leaf squares treated with the total extract (c, d), and difference of residence time on leaf squares treated with the distillation residue from that on leaves treated with the vacuum distillate (e) in choice bioassays. Vertical lines with plots indicate ± SE. Asterisks indicate significant difference (Wilcoxon's signed rank test, $P < 0.05$).

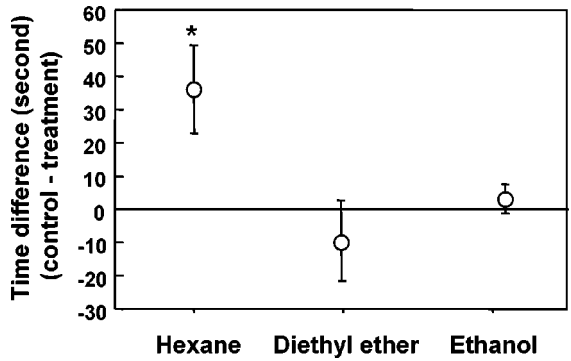

FIG. 3. Effect of hexane, diethyl ether, and ethanol-eluted fractions, prepared by liquid chromatography through Florisil, of adult *Coccinella septempunctata* vacuum distillation residue on residence times of *Aphidius ervi* females on broad bean leaf squares. Data are expressed as differences from residence times on control leaf squares in choice bioassays. Vertical lines with plots indicate ± SE. Asterisks indicate significant difference (Wilcoxon's signed rank test, $P < 0.05$).

TABLE 1. COMPOUNDS IDENTIFIED FROM COUPLED GC-MS ANALYSIS OF SEVEN-SPOT LADYBIRD, *Coccinella septempunctata*, EXTRACTS

Peak number[a]	Compound	Percentage (%)[b]
1	*n*-Tricosane	9.5
2	*n*-Pentacosane	4.9
3	7,11-Dimethylpentacosane	3.1
4	*n*-Heptacosane	4.2
5	13-Methylheptacosane	27.1
6	9-Methylheptacosane	
7	7-Methylheptacosane	
8	9,13-Dimethylheptacosane	26.8
9	7,11-Dimethylheptacosane	
10	7,11,15-Trimethylheptacosane	10.0
	Other (unidentified)	14.4

[a] Peak numbers correspond to labeled peaks in GC-MS traces in Figures 4a, b, c, and d.
[b] Percentage based on peak area calculated by integration on GC of adult body extract.

To identify the chemicals responsible for parasitoid avoidance, the biologically active hexane-eluted fraction was analyzed by high resolution GC and coupled GC-MS. The sample was composed almost entirely of aliphatic hydrocarbons (Table 1; Figure 4a). Components identified included *n*-tricosane, *n*-pentacosane, and *n*-heptacosane, which were identified by comparison with published MS spectra (NIST, 1990) and peak enhancement on GC using authentic samples. Other major components were identified tentatively as branched hydrocarbons from their characteristic fragmentation patterns and by comparison with MS data from similar studies elsewhere (Kosaki and Yamaoka, 1996; Hemptinne et al., 2001). Similar arrays of straight-chained and branched hydrocarbons were found in the hexane-eluted fraction obtained from extracted *C. septempunctata* larvae, and from footprint extracts collected in glass Petri dishes in which *C. septempunctata* male and female adults had been allowed to walk (Figure 4b, c, and d, respectively). Solutions of the straight-chained hydrocarbons in ethanol, at the levels found in the adult ladybird extract, were prepared for bioassays. Leaf squares sprayed with *n*-tricosane and *n*-pentacosane were significantly avoided by *A. ervi*, but *n*-heptacosane did not induce avoidance behavior (Figure 5). A two-way ANOVA showed that both *n*-tricosane and *n*-pentacosane induced avoidance behavior (for *n*-tricosane, $F = 12.38$, 1 and 76 df, $P < 0.01$; for *n*-pentacosane, $F = 8.00$, 1 and 76 df, $P < 0.01$), and reduced residence time on treated leaf squares (Figure 6). The interaction between these two chemicals was not significant (*n*-tricosane $\times$ *n*-pentacosane, $F = 0.064$, 1 and 76 df, $P = 0.80$), indicating that their effects on parasitoid avoidance responses were similar. A mixture of these two hydrocarbons significantly reduced the level of parasitism on treated broad bean plants,

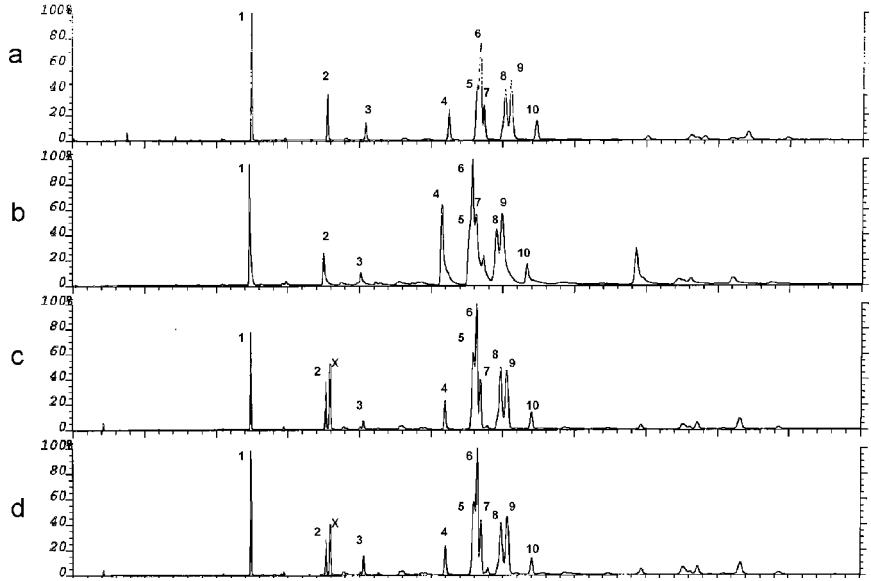

FIG. 4. Coupled gas chromatography-mass spectrometry (GC-MS) analysis of seven-spot ladybird, *Coccinella septempunctata* extracts: (a) adult whole body extract, hexane-eluted fraction; (b) larvae whole body extract, hexane-eluted fraction; (c) adult male footprint extract; and (d) adult female footprint extract. Peak numbers correlate to identifications listed in table. X = dioctyl phthalate.

compared with untreated controls, although the effect disappeared within 24 hr (Figure 7).

DISCUSSION

To our knowledge, this study is the first identification of semiochemicals that mediate intraguild predator avoidance. The aphid parasitoid, *A. ervi*, avoided low-volatile compounds in chemical trails deposited on leaf surfaces by both adults and larvae of *C. septempunctata* ladybirds. However, components present in the trails retained biological activity during relatively short periods, and parasitoids did not avoid areas treated 24 hr previously. From the active fraction isolated by liquid chromatography, *n*-tricosane and *n*-pentacosane were identified as the main active stimuli, inducing intraguild predator avoidance by this parasitoid. These two hydrocarbons negatively affected parasitism rates of pea aphids on broad bean plants, but the duration of activity was also less than 24 hr.

n-Tricosane and *n*-pentacosane have been reported previously as components of trails of seven-spot ladybirds that occur in Japan (Kosaki and Yamaoka, 1996).

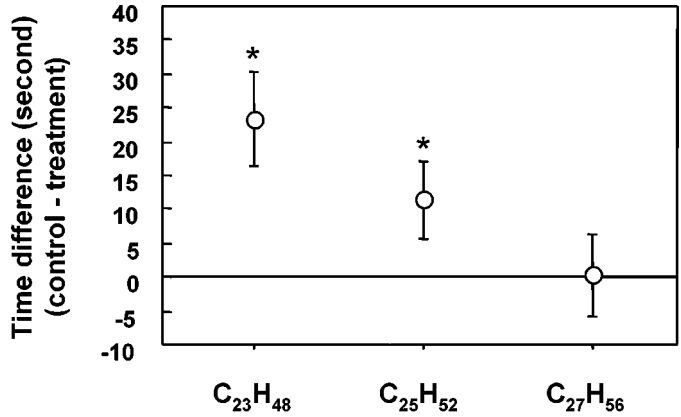

FIG. 5. Effect of *n*-tricosane ($C_{23}H_{48}$), *n*-pentacosane ($C_{25}H_{52}$), and *n*-heptacosane ($C_{27}H_{56}$) on residence times of *Aphidius ervi* on broad bean leaf squares. Data expressed as differences from residence times on control leaf squares in choice bioassays. Vertical lines with plots indicate ± SE. Asterisks indicate significant difference (Wilcoxon's signed rank test, $P < 0.05$).

In the same study, it was shown that the hydrocarbons in trails were identical to cuticular surface chemicals, and large amounts of these chemicals were also secreted from the tarsi. These findings, along with our results, indicate that *A. ervi* avoids *n*-tricosane and *n*-pentacosane in the "footprints" of seven-spot ladybirds,

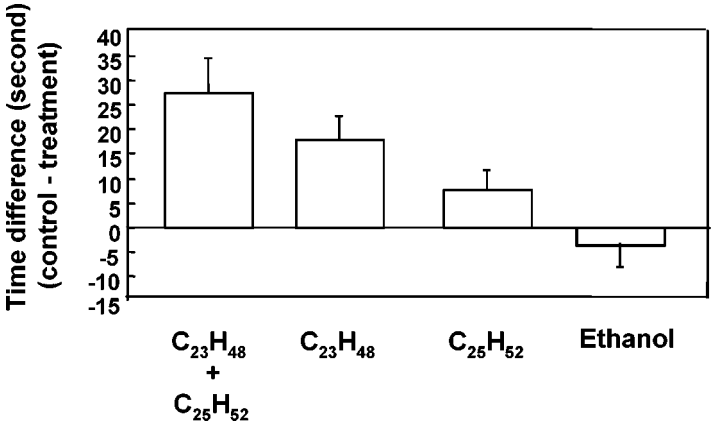

FIG. 6. Differences in residence times of *Aphidius ervi* females on a leaf disc treated with *n*-tricosane ($C_{23}H_{48}$), *n*-pentacosane ($C_{25}H_{52}$), a *n*-tricosane/*n*-pentacosane mixture (treatment), and ethanol (control) in choice situations. Vertical lines indicate ±1 SE (two-way ANOVA).

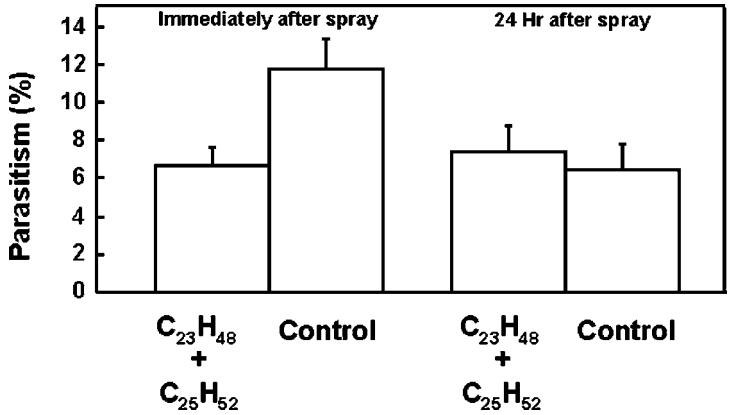

FIG. 7. Differences in percentages of parasitism by *Aphidius ervi* on broad bean seedlings sprayed with a *n*-tricosane/*n*-pentacosane mixture in ethanol or with ethanol only (control), immediately and 24 hr after release of a parasitoid female in an experimental arena with both types of seedlings. Vertical lines indicate ± SE (Wilcoxon's signed rank test, $P < 0.01$).

and that these two compounds additively affect avoidance responses. Mixtures of hydrocarbons, of which *n*-pentacosane is the major component, are also present in larval trails of the two-spot ladybird, *Adalia bipunctata*, and it has been shown that the mixture functions as an oviposition-deterring pheromone for *A. bipunctata* (Hemptinne et al., 2001). Our findings suggest that *A. ervi* may also use hydrocarbons to avoid trails of *A. bipunctata*.

The results in this study are consistent with previously published work, in that ladybird trails have a relatively short period of activity (<24 hr) (Nakashima and Senoo, 2003). Even though *n*-tricosane and *n*-pentacosane are relatively stable chemicals, their activities disappear quickly. This may be due to absorption or dilution of the hydrocarbons into the plant cuticular lipid layer, or even evaporation/sublimation from the leaf surface over time, as even higher molecular weight hydrocarbons are known to possess vapor pressures at ambient temperatures.

The use of semiochemicals in predator avoidance can be advantageous to parasitoids, because chemicals deposited by *C. septempunctata* give parasitoids both spatial and temporal information on predator presence. Information from predator trails is a reliable indicator of areas where intraguild predators are located, and this spatial information is vital for parasitoids in reducing the risk of predation, especially from aphidophaous ladybirds, as larval ladybirds are likely to stay and complete development within an aphid patch (Dixon, 2000). Additionally, temporal information may also be important for parasitoids. Contrary to larval ladybirds, adults of *C. septempunctata* are highly mobile, and it has been estimated that the average residence time of adult ladybirds in an aphid patch lasts for several hours

(van der Werf et al., 2000). Thus, the limited activity (<24 hr) of ladybird trails is advantageous to parasitoids, because they are allowed to forage in patches no longer occupied by adult ladybirds. However, larval ladybirds would continuously renew the chemical signals while continuing to forage in a single aphid-infested patch.

Coccinella septempunctata displays aposematic warning coloration and is highly toxic, although its own parasitoid, *Dinocampus coccinellae*, uses the free base, toxic alkaloid precoccinelline as a kairomone (Al Abassi et al., 2001). There could, therefore, be an element of toxin avoidance in the case of *A. ervi*, using the hydrocarbons as an extension of the aposematic cues deployed by *C. septempunctata*. However, such a general effect of these particular compounds is unlikely as 2-isopropyl-3-methoxypyrazine is known to provide this signal as well as having a role as an aggregation pheromone for this particular ladybird (Al Abassi et al., 1998).

Intraguild predator avoidance would reduce the likelihood of IGP, and this has been suggested in predator–predator (Pallini et al., 1998; Schellhorn and Andow, 1999) and predator–parasitoid (Taylor et al., 1998; Raymond et al., 2000) interactions. In the *A. ervi–C. septempunctata* system, *n*-tricosane and *n*-pentacosane function as kairomones for *A. ervi* probably because these chemicals help them to reduce predation risks to their progeny. The avoidance responses of parasitoids mediated by kairomones can reduce foraging opportunities in patches that contain intraguild predators, and rates of parasitism may decrease in these aphid patches. Thus, intraguild predator avoidance may be a factor affecting parasitoid population dynamics, and may affect aphid population suppression. The commercially available chemicals, *n*-tricosane and *n*-pentacosane, which are the active compounds in ladybird trails, may be useful for the enhancement of parasitoid roles in biological control programs by helping to concentrate them in target areas through reduction of unproductive foraging in nontarget areas. Although it is possible to develop materials and formulations that will extend the short effective period of such compounds, initial field evaluation of the effects of the semiochemicals on parasitism rates is required to assess fully their impact and potential.

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MONOTERPENES AND EPICUTICULAR WAXES HELP FEMALE AUTUMN GUM MOTH DIFFERENTIATE BETWEEN WAXY AND GLOSSY *Eucalyptus* AND LEAVES OF DIFFERENT AGES

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Abstract—The autumn gum moth, *Mnesampela privata*, is a native Australian species whose preferred host, *Eucalyptus globulus* (Myrtaceae), is an aromatic evergreen tree that has long-lived waxy leaves during the juvenile phase of growth. We compared the behavioral and antennal responses of female moths to whole leaves (new and old) and samples of foliar chemicals (from new and old leaves) from a typical *E. globulus* subsp. *pseudoglobulus* with responses to a glossy, half-sibling *E.g.* subsp. *pseudoglobulus* putative hybrid (the result of natural cross-pollination). We also studied larval survival and development on leaves from the same trees. In laboratory binary-choice assays, female *M. privata* laid more eggs on waxy leaves than on glossy leaves thereby confirming the nonpreference for the glossy tree that was observed in the field. Analyses of the monoterpenes and waxes of both trees revealed that they had comparable suites of monoterpenes and total oil contents but different suites of epicuticular waxes. Headspace extracts differed in the intensity of component monoterpenes. Gas chromatographic analyses with electroantennographic detection showed different patterns of monoterpene detection. Leaves of the glossy tree had a less diverse array of epicuticular waxes than those of the waxy tree. Electroantennographic screening of responses to wax extracts from leaves (new and old) from either tree revealed positive dose-dependent responses of female

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antennae to waxes from new leaves only. Binary-choice assays also revealed a strong preference by ovipositing females for new, compared to old, leaves whether they were from the waxy or the glossy tree. However, new leaves from either tree could be manipulated (by physical abrasion of epicuticular waxes) so that females would lay almost no eggs on them. Larval survival did not differ between groups reared on leaves from both trees (new and old). Over 70% of all larvae survived to pupation. However, larvae reared on leaves from the glossy tree took longer to pupate than those reared on leaves from the waxy tree. Also, larvae reared on new leaves from either tree did not perform as well as those reared on old leaves. Monoterpene and wax cues are suggested as helping female *M. privata* locate preferred hosts in native forests.

Key Words—Age-related leaf traits, GC-EAD, EAG, oviposition, larval performance, autumn gum moth, *Mnesampela privata*, *Eucalyptus globulus*, monoterpenes, waxes.

INTRODUCTION

For leaf-eating insects endemic to vegetationally complex habitats, finding preferred host plant species is presumably a difficult task. Among factors that may potentially confound the location of a preferred host plant are cues from similar species. This issue may be especially important in Australian forests where species of *Eucalyptus* L'Hér. (Myrtaceae) dominate. Eucalypts seldom form monospecific stands; typically they comprise locality-specific associations of anywhere between 2 and 10 species per hectare (Wardell-Johnson et al., 1997). Because the essential oils of different species often comprise the same monoterpenes (Boland et al., 1991), a particular species' odor may share components typical of one or more other species. Tahvanainen and Root (1972) suggested that preferred plant species may achieve some degree of "associational resistance" by being close to nonhosts because "odor-masking" reduces the chances of location by herbivorous insects. Despite the potentially huge implications of vegetational complexity for the ecology of an insect herbivore, no study has attempted to elucidate the behavioral and physiological mechanisms that enable a particular insect species to make these crucial choices in a eucalypt-dominated environment. Several studies have touched upon mechanisms used by eucalypt-feeding insects to achieve host specificity and facilitate discrimination between hosts. Edwards and Wanjura (1990) suggested that unspecified behavioral and physical adaptations allowed *Chrysophtharta m-fuscum* (Boheman) (Coleoptera: Chrysomelidae) to specialize on more waxy *Eucalyptus* species. Edwards et al. (1993) reported that the severity of defoliation of individual *Eucalyptus* of the same species by *Anoplognathus* Leach (Coleoptera: Scarabaeidae) was positively correlated with the concentration of 1,8-cineole in leaves. Although these authors did not suggest that 1,8-cineole was necessarily the biologically active compound, it was assumed that olfaction of terpenoids deters feeding on trees whose leaves are higher in these compounds. Once a female has

landed on a preferred species, additional assessment of the host may be required to ensure that the leaves on which she will oviposit will allow better survival and development of her offspring than might nearby leaves of the same host (Renwick, 1989; Renwick and Chew, 1994).

Species of perennial plants endemic to Mediterranean- and tropical-climate ecosystems, such as trees in the genus *Eucalyptus*, are characterized by their sclerophyllous foliage (Morrow, 1983; Specht and Moll, 1983; Turner, 1994; Coley and Barone, 1996). The low annual rainfalls and soil nutrient levels of some of these ecosystems have led to the evolution of plants whose leaves exhibit high specific leaf weight (SLW) or leaf toughness (Cunningham et al., 1999). High SLW is a trait associated with sclerophylly. Although sclerophylly is probably an adaptive response to abiotic factors, Lucas et al. (2000) wrote that its efficacy in "preventing herbivory is indisputable." As a probable consequence, many herbivorous insects specific to plants with sclerophyllous foliage have a tendency to seek out new, softer leaves (Morrow, 1983; Ohmart, 1991; Ohmart and Edwards, 1991; Coley and Barone, 1996; Steinbauer et al., 1998). This is probably because the new leaves are the most easily ingested (because of low SLW) and provide the best nutrition, especially for neonates (Ohmart et al., 1987; Aide and Londoño, 1989; Howlett et al., 2001; Nahrung et al., 2001). Low SLW may also be the mechanism behind some insect species' preference for softer leaf types, e.g., morphologically juvenile leaves (Steinbauer, 2002). To date, no author has suggested a mechanism that explains how nonherbivorous life cycle stages (e.g., those with haustellate mouthparts) make the choice between a new or an old leaf of any morphological type.

The leaves of many eucalypt species, especially some juvenile leaves, undergo pronounced changes in glaucousness or wax bloom as they age. New juvenile leaves are highly glaucous, but the wax layer gradually diminishes as the leaf ages. We are not referring to the changes in waxiness that occur as eucalypt leaves progress through juvenile and adult morphologies, i.e., adult eucalypt leaves are inherently less waxy than juvenile leaves (Li et al., 1997). Instead, we are referring to the process of wax loss as all leaves grow older. The gradual loss of epicuticular waxes is not unique to eucalypts. For example, the amount of wax on the blades of two varieties of sorghum was found to decline as plants aged (Atkin and Hamilton, 1982). In other plant species, the loss of epicuticular waxes has been shown to occur via either physical abrasion (e.g., via abiotic factors such as rain) or chemical changes in amounts of component compounds (Baker and Hunt, 1981). Similar changes have also been reported from peach leaves (Baker et al., 1979). Hence, age-related changes in epicuticular waxes would seem to provide a reliable indirect measure of leaf quality for phytophagous insects. A cue that can be detected on contact would seem essential for nonherbivorous life cycle stages. Moreover, because the development of epicuticular waxes can be influenced by soil moisture, solar radiation, and ambient temperature, they may also provide insects with cues

indicative of a plant's condition (Baker, 1974; Thomas and Barber, 1974; Baker and Procopiou, 1980).

Using a putative hybrid that had reduced epicuticular waxes (result of natural cross-pollination) compared to its waxy half-siblings, we investigated the possible roles of monoterpenes and waxes from different trees on oviposition (both in the field and in laboratory binary-choice assays) and antennal responses of female *Mnesampela privata* (Guenée) (Lepidoptera: Geometridae). We also used leaves of different ages to study the effect of age-related loss of waxes on female responses. We investigated the implications of female choices upon the development of larvae. We were particularly interested in the possibility that females use epicuticular waxes as a way of assessing leaf age.

METHODS AND MATERIALS

Study Organisms

Insects. Adult *M. privata* are medium to large (wingspans: 38–44 mm, females usually slightly larger), nocturnal moths that hold their wings roof-wise over the body when at rest (McQuillan, 1985; pictured in McFarland, 1988). On *Eucalyptus globulus*, eggs are laid exclusively on the undersides (which are waxier than the adaxial surfaces) of juvenile leaves in clutches that can range in number from just a few eggs to well in excess of 300 eggs (Steinbauer et al., 2001; Steinbauer, 2002). On species of eucalypt where there is no apparent difference in the waxiness of either leaf side (e.g., *E. nitens* and *E. rubida*), eggs can be laid on both leaf surfaces (Lukacs, 1999; M. J. Steinbauer, 1998–2003, personal observation). Depending upon ambient temperature and relative humidity, larvae will emerge approximately 14 days after being laid (McFarland, 1973; McQuillan, 1985; Lukacs, 1999). Neonates do not consume their eggshells after emerging, preferring to begin feeding on the leaf on which they were deposited (McQuillan, 1985). Neonates and early instars largely remain in sibling groups and feed externally on intervein leaf tissues, i.e., they deliberately avoid all veins and oil glands and will eventually skeletonize the natal leaf. Avoidance of vascular structures reflects a preference for the least tough parts of leaves (Steinbauer and Matsuki, unpublished data). Older larvae may remain in small aggregations or live singly and feed by ingesting whole pieces of leaf, only avoiding the midrib (Steinbauer and Matsuki, unpublished data). Coincident adult and offspring generations and a preference on the part of females for high-quality hosts are traits associated with species that have latent population dynamics (Price, 2003). Fifth instars especially are highly mobile and can disperse large distances before burrowing into the soil to pupate; they may or may not enter a pupal diapause (Lukacs, 1999; Steinbauer et al., 2001).

Eucalypts. Trees of *Eucalyptus globulus* subsp.*pseudoglobulus* (Naudin ex Maiden) Kirkpatr. were planted at Ginninderra Experiment Station, Australian

Capital Territory (35°09′S, 149°02′E; 615 m ASL), between October 29 and November 2, 1998. The trees were grown from seed collected by the Department of Conservation and Natural Resources of Victoria and supplied by the Australian Tree Seed Centre. Seed came from a single wild mother and was produced by natural cross-pollination. That is, these trees represent a family of the Murrungower Road provenance (Victoria; 37°37′S, 148°43′E; 400 m ASL). All trees were producing juvenile foliage throughout the studies. One of them had a unique leaf morphology and glossy (terminology after Eigenbrode and Espelie, 1995) phenotype when compared to its half-siblings, and was possibly a hybrid (see next). Leaves from the glossy phenotype tree, as well as a randomly selected normal half-sibling, were examined using light and scanning electron microscopy (SEM) to record the differences between their appearances and epicuticular waxes.

Because there was only one glossy tree at our site, one of the other 28 half-siblings was chosen at random for comparative studies of insect responses to leaves typical of both genotypes. Given the geographic location of the mother tree, information about the species of *Eucalyptus* with which *E. globulus* Labill. will naturally hybridize (see Strauss, 2001), as well as the phenotype of the glossy tree, it seems likely that the pollen that fertilized this particular seed came from *E. cypellocarpa* L. Johnson. However, the genotype of the hybrid tree could not be precisely resolved by using microsatellite analysis (R. Jones, B. Potts, and R. Vaillancourt, 2001, personal communication). Consequently, we refer to this tree as a putative hybrid. Unfortunately, no *E. cypellocarpa* occur in the vicinity of Canberra. This precluded this species' use in our assays.

Leaf Age and Toughness. We defined *new leaves* as current season leaves, and *old leaves* as leaves from the previous growing season. The scale leaf marks the point where the current season leaves began expanding. SLW of 12 individual leaves, as estimated with a 24 mm^2 hole punch, was used as the index of leaf toughness. Estimation of SLW also allows leaf water content to be determined. SLW increases as leaves age and/or expand (Steinbauer, 2001).

Incidence of Mnesampela Privata in the Field. The incidences of *M. privata* on 28 waxy and 1 glossy phenotype trees were recorded for two seasons. Surveys involved 1-min whole-tree observations of each *E. g., pseudoglobulus* at Ginninderra Experiment Station during which the presence/absence of eggs, larvae, and moths were recorded.

Oviposition Choice Assays

Pupae were reared from eggs from a laboratory culture of *M. privata* that was begun anew annually. Larvae were reared in a 20°C controlled temperature room (range, 20.4–21.2°C), with a 12:12 hr reverse-cycle photoperiod and ambient relative humidity (range, 38–84%). Pupae were placed into cages when they were within 1–2 days of eclosion. Assays were conducted in 54 × 37 × 28 cm cages with

1.0 × 0.5 mm nylon gauze. Each cage had one container of 5% sugar water with a protruding cotton wick. Two branchlets of foliage (binary-choice) were kept in water in diagonally opposite corners of each cage; leaves did not overlap. Assays were run for 7 days. All assays were conducted in the same constant temperature room in which larvae were reared. Moths were used only once.

Four types of assay were conducted. Assay 1 compared oviposition on waxy vs. glossy phenotypes, regardless of leaf age, using 11 cages with three females per cage. Assay 2 evaluated oviposition on old vs. new leaves of different phenotypes, using six cages with one female per cage. Assay 3 tested female preference for old vs. new leaves of the same phenotype, using nine (for waxy) or four (for glossy) cages with one female per cage. Finally, assay 4 examined the effect of presence vs. absence of the entire wax layer on oviposition choice on new leaves of the same phenotype, using three (for waxy) or four (for glossy) cages with one moth per cage. Selective removal of leaves was used to create branchlets displaying only the desired leaf age (method used for assays 2, 3, and 4). Epicuticular waxes were removed (*de-bloomed*) from both sides of all leaves (used only for assay 4) by gentle abrasion with soft tissue paper.

At the conclusion of each assay, all leaves were examined, the number of eggs was counted, and leaves were excised for drying. The areas of oven-dried and flattened leaves were obtained by using a leaf area meter. Analysis of covariance (ANCOVA) was used to compare log (eggs + 1) transformed data from each assay. Total leaf area (i.e., leaf area multiplied by 2) was used as a covariate.

Larval Survival and Development Assays

Survival of larvae from egg to prepupae and the weights of pupae from each host were studied. Rearing conditions were the same as those for the oviposition choice assays. Four separate groups of five eggs were placed onto leaves of each of the four types. Groups of larvae were examined at 3, 7, 10, 13, 17, 21, 25, 28, 32, 35, 38, 42, 50, 57, 67, and 73 days after eggs were first placed onto leaves. On these days, larval mortality and the numbers of individuals reaching the prepupal or pupal stage were recorded. Fresh leaves were given to larvae as required. Following pupation, individuals were removed from their silk cocoons for sexing and measurement of live weights. The live weights of most pupae were obtained when individuals were between the pigment-spots to black-eyes stages of development. Pupal dry weights were estimated by using the water contents of 10 pupae of each sex that had been reared previously and then dried at 70°C until constant weight.

We used Kaplan–Meier analysis to compare survival of larvae reared on different hosts (Kaplan and Meier, 1958). Survival estimates for selected pairs of Kaplan–Meier survival curves were compared by using log-rank tests (Cox, 1972). Developmental durations of larvae and estimated pupal dry weights were

compared with General Linear Model analysis (interaction terms were removed from models when not statistically significant) and *post hoc* one-way ANOVA.

Composition of Monoterpenes

We used three techniques to extract foliar monoterpenes. Leaves were harvested haphazardly, placed into sealed plastic bags, and kept on ice until returned to the laboratory.

Monoterpene Extraction with Cold Solvent. To estimate the percentages of component monoterpenes, four current season's leaves from each tree were soaked for 22 hr in 10 ml of dichloromethane at room temperature. Extracts were analyzed using an Hewlett-Packard 5890 gas chromatograph coupled to an Hewlett-Packard 5970B Mass Selective Detector via an open-split interface. The column was a 25 m $\times$ 0.32 mm HP-1 (0.17 μm), operated with helium as carrier gas with a head pressure of 10 psi. The initial gas velocity was 40 cm/sec (N.B. This changed according to the temperature of the column.). The oven temperature was 30°C for 2 min and then set to rise to 240°C at 6°C/min. The range from m/z 35 to m/z 400 was recorded at 2 scans per sec. Injections of leaf extract were 2 μl, with a split of 10:1 on injection. The injection port temperature was 220°C and the transfer line was at 260°C.

Monoterpene Extraction with Hot Solvent. To estimate the total oil content of leaves, 100 mg of fresh slivers from each of two leaves were placed into a glass tube. Slivers were immersed in 400 μl of HPLC grade hexane containing 100 ppm tridecane as an internal standard. The glass tube was then sealed and placed into a 100°C oven for 1 hr. After this, the glass tubes were opened, and the solvent extract was pipetted into 12 $\times$ 32 mm clear vials (Alltech Assoc. Inc., Deerfield, USA). Vials containing extracts were diluted with hexane ready for gas chromatography. Leaf extracts were then assayed by gas chromatography using a Varian Star 3400 GC with Varian 8200 autosampler. We joined together an Alltech Econocap and a Zebron^{TM} ZB-1701 (30 m $\times$ 0.32 mm $\times$ 0.25 μm film with a 14% cyanopropylphenyl-methylpolysiloxane liquid phase) column for these analyses. Helium at 20 psi was the carrier gas. One-microliter quantities of leaf extract were automatically injected into the column. The oven temperature was set initially at 50°C (held for 1 min), before rising to 80°C (held for 12 min) at 50°C per min, and then set to rise again to 240°C (held for 10 min) at 10°C per min. The duration of each run was 39.6 min. The flame ionization detector was set at 300°C. The total oil content of leaves was calculated by applying the flame ionization detector (FID) response factor of the standard (tridecane) to all components. Total oil contents were compared using two sample t tests assuming unequal variance and the two-tailed critical value of the t statistic.

Monoterpene Extracts of Headspace Odors. Headspace volatiles were sampled from two branchlets bearing approximately similar numbers of leaves.

Branchlets were cut from each tree and taken as quickly as possible to the laboratory where they were placed immediately into water. Branchlets were enclosed within microwaveable polyacetate oven bags that were tightly gathered around the base of the stem with tape. One glass tube containing 5 mg of Porapak Q, held in place by glass wool plugs, per branchlet was simultaneously inserted between the folds of the bag. Air was pulled out of the bags by a battery-operated vacuum pump at a rate of 60 ml/min. Laboratory air was sampled on a control filter. Air was removed from each bag for 2 hr, after which volatiles were eluted from the Porapak Q with pentane. Samples were stored in a freezer and used for the GC-EAD analyses detailed below.

Headspace extracts were analyzed by solid phase micro extraction after removal of the bulk solvent (Boyle et al., 2002). A 100-μm polydimethylsiloxane fiber (Supelco, Supelco Park, Bellefonte, PA) was used. This was equilibrated with the sample for 10 min before desorption at 200°C for 2 min in the GC injection port (procedure for chemical analysis as per "solvent extraction" above).

Composition of Epicuticular Waxes

Survey of Wax Components. Leaf waxes were extracted from 2 g of fresh leaf cut into approximately 1 cm^2 discs, which were then soaked in 10 ml of HPLC grade hexane for 50 min with occasional gentle stirring. This solution was decanted and analyzed directly by combined gas GC-MS on an HP 5890GC coupled via an open-split interface to an HP 5970B Mass Selective Detector. One-microliter injections were made in the splitless mode, with an injection temperature of 300°C and transfer line temperature of 290°C. The column was a 25 m $\times$ 0.32 mm HP1 (0.17-μm film thickness), with a temperature program of 60°C (held for 1 min) to 300°C at 10°C/min, with an 8-min hold time at the final temperature. Mass spectra were collected over the range m/z 40 to m/z 550 at two scans per sec. Individual components were identified from an in-house library of relevant mass spectra in combination with relative retention times. Estimations of percentages of individual compounds were based on contribution to the total ion current.

Amounts of Individual Wax Components and Quantity of Wax per Unit Leaf Area. Leaf discs (165 mm^2) were punched from 10 fresh leaves of each age and each phenotype. Leaf discs were immersed in 2 ml of dichloromethane in individual glass vials that were gently shaken for 20 sec prior to the removal of the disc with clean metal forceps. To each vial, 80 μg of *n*-tetradecane added to act as an internal standard. The relative amounts of a suite of readily identified components in each of these extracts were estimated by using combined GC-MS with Selected Ion Monitoring (SIM) of diagnostic ions. Peaks that were originally identified from full scan GC-MS data of the stronger samples (e.g., samples from waxy new leaves) were quantified in all samples on the basis of these diagnostic ions. For the SIM analyses, diagnostic ions were monitored within appropriate time windows,

with a dwell time of 50 msec/ion. The ions used were as follows: m/z 85 for tetradecane, m/z 74 for methyl eicosanoate, m/z 91 for all benzyl esters, m/z104 for all phenylethyl esters, m/z 326 for eucalyptin, and m/z 100 for all β-diketones. The resulting peak areas were corrected to calculate the total ion current, on the basis of the proportion that each diagnostic ion was of a "clean" mass spectrum of each component. As it was not possible to determine absolute weights for all components because of the lack of synthetic standards, the final results were expressed as micrograms of tetradecane equivalents per square millimeter of leaf area. One-way ANOVA and *post hoc* tests were used to make comparisons between compounds according to host and leaf age.

To get a rough estimate of the quantity of wax per leaf type, twelve 600 mm^2 (for seven glossy new leaves only) or 800 mm^2 rectangles were cut from 12 freshly harvested new and old leaves of each of the glossy and waxy phenotype trees, respectively. Three ml of dichloromethane were poured over each side of each leaf rectangle in 1-ml aliquots. Each aliquot of dichloromethane and dissolved epicuticular waxes was collected in the same preweighed glass Petri dish. Petri dishes with epicuticular wax residues were reweighed after evaporation of dichloromethane, and the amount of wax was estimated by difference.

Electrophysiological Studies

Antennal Preparations. The tip of an excised female antenna was cut off, and the antenna was mounted on an antennal holder with electrode gel. The electrode holding the base of the antenna was grounded. The distal end was connected via an interface box (Syntech, Hilversum, the Netherlands) to a PC for recording of the EAG signal (see Schiestl and Marion-Poll, 2002). Two antennae were used in parallel for each recording. Purified and humidified air was directed over the antennal preparation.

Antennal Stimulation with Foliar Monoterpene Odors and GC-EAD. Three-μl aliquots of headspace extracts were injected on column into a Varian 3400 GC. The temperature profile began at 40°C (held for 1 min) and increased to 300°C (held for 20 min) at a rate of 10°C/min. The GC was equipped with an EC-1 column (30 m $\times$ 0.25 mm). Helium was used as the carrier gas. A SGE GC effluent splitter (split ratio, 1:1) was used, and the redirected volatiles were added to a purified and humidified air stream passing over the antennae. EAD signals and FID responses were simultaneously recorded.

We performed 5–10 GC-EAD replicate runs for each sample and checked the reproducability of all reactions to differentiate electroantennographic responses from noise. GC-EAD active compounds were identified by cross-referencing with the GC-MS analyses obtained by using the headspace extracts, as well as coinjection with some reference compounds, namely myrcene, γ-terpinene (Sigma Chemical Co., MO), terpinene-4-ol, α-terpineol, terpinolene, α-pinene, 1,8-cineole, *p*-cymene, and limonene (in-house standards).

Antennal Stimulation with Epicuticular Wax Odors and EAG. Extracts for use in the antennal stimulation studies were obtained by pouring 2 ml of dichloromethane over the abaxial side of each of five fresh leaves of each age and each phenotype. Extracts from individual leaves of the same age and phenotype were accumulated in the same glass vial. Five, 10, 25, and 50 μl of sample were applied onto a filter paper in a pipette tip. A control filter paper was treated with 50-μl solvent only. Enough time was allowed for the solvent to evaporate. During stimulation, 2.5 ml of air were puffed over the filter paper and into the air-stream directed over the antenna. Stimulations were applied with minimum 20-sec intervals, starting each time with the control, and then different sample concentrations, either from least to strongest concentration or in reverse order in equal frequency. For each antenna, the control and each concentration was tested 2–4×.

To normalize and compensate for artifacts of mechanoreception, EAG responses (−mV) were expressed as percent response of the control stimulant (i.e., background air blown over the antennae). From these values, mean responses to each concentration were calculated for each individual insect. Response data less than 100% means that antennae responded less to the stimulus than they did to background air only. A one-way ANOVA and pair-wise comparisons were used to compare responses.

RESULTS

Appearance and Toughness of Glossy and Waxy Leaves. Glossy tree leaves were comparatively small and distinctly green on both sides (Plate 1A). The leaves of the waxy half-sibling were bluish green from above and paler underneath due to a dense layer of epicuticular waxes (Plate 1A). The waxes on the abaxial sides of glossy leaves are less dense and appear as discrete tubes (Plate 1B and D). The stomata on glossy leaves are visible. The abaxial sides of waxy leaves are covered in a dense layer of wax tubes (terminology after Hallam and Chambers, 1970; Meusel et al., 2000), many of which appear to have coalesced (Plate 1C and E).

Glossy new leaves were of significantly lower SLW (0.12 ± 0.002 mg mm^{-2}; $M \pm$ SE, here and throughout the text) than any other leaf type or age (waxy new 0.14 ± 0.004 mg mm^{-2}, waxy old 0.15 ± 0.005 mg mm^{-2}, and glossy old 0.15 ± 0.003 mg mm^{-2}). This is as expected given the generally smaller size of these leaves. Increases in SLW associated with increasing leaf age were associated with decreased leaf water contents. The water contents of the new leaves from both trees were comparable and, on average, higher (waxy new 53.7 ± 0.4% and glossy new 55.6 ± 0.3%) than those recorded for old leaves from both trees (waxy old 52.8 ± 0.6% and glossy old 52.4 ± 0.5%).

Incidence of Mnesampela Privata in the Field. Over two seasons, four separate instances of eggs and larvae on the waxy phenotype tree were recorded. No eggs or larvae were recorded on the glossy phenotype tree during the routine whole tree

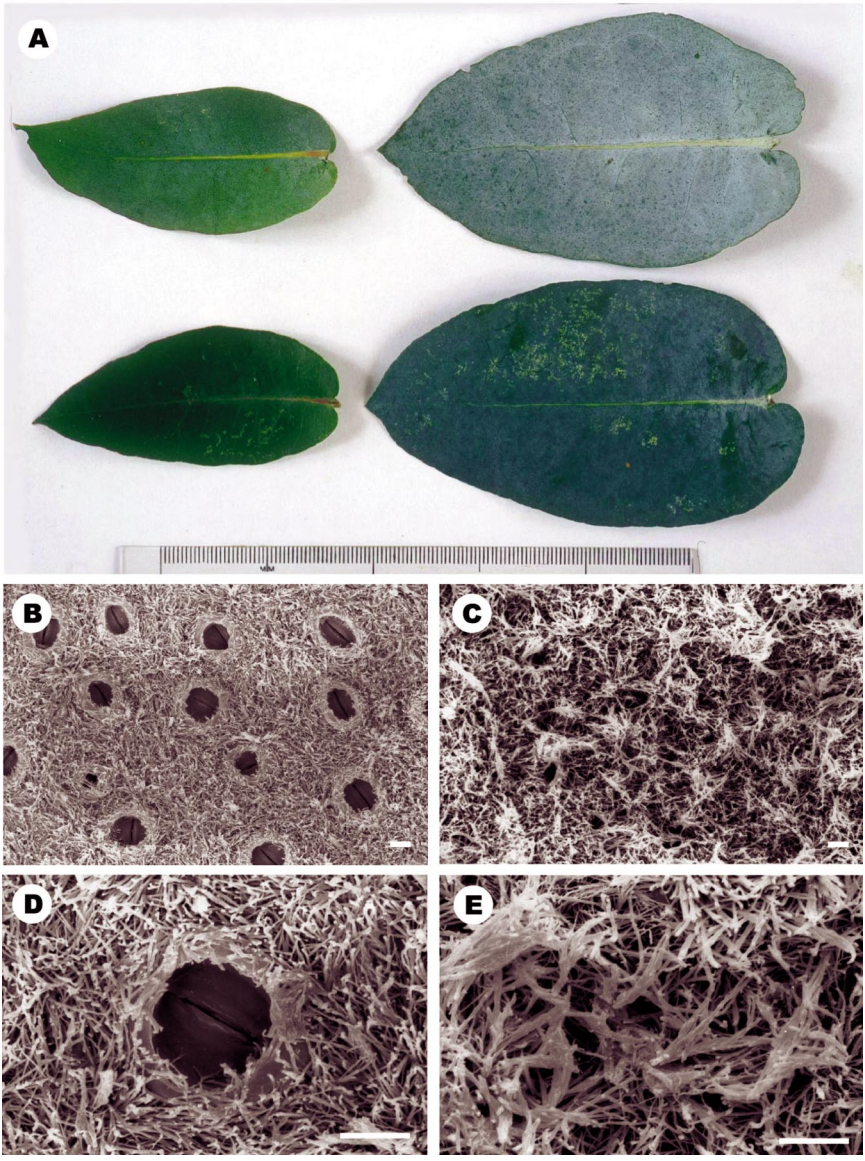

PLATE 1. (A) Whole views from abaxial (top of image) and adaxial (bottom of image) sides, respectively, of glossy (left) and waxy (right) leaves, respectively (scale mm), and SEMs of the epicuticular waxes on the abaxial sides of glossy leaves (B and D) and on waxy leaves (C and E), respectively (scale bars = 10.0 μm). Stomata are not visible on waxy leaf at either magnification.

TABLE 1. INCIDENCE OF *M. privata* ON WAXY AND GLOSSY PHENOTYPE TREES

Season and date	Waxy ($N = 1$)	Glossy ($N = 1$)	Other waxy trees ($N = 27$)
First season			
March 24, 1999	—	—	11.1% (eggs and larvae)
April 7, 1999	x (larvae)	—	24.0% (eggs and larvae)
April 21, 1999	x (eggs)	—	25.9% (larvae)
May 5, 1999	—	—	25.9% (larvae)
May 18, 1999	—	—	14.8% (larvae)
Second season			
December 15, 1999	—	—	0.0%
January 20, 2000	—	—	18.5% (larvae)
February 16, 2000	—	—	11.1% (eggs and moth)
March 2, 2000	—	—	18.5% (eggs, larvae, and moth)
March 30, 2000	x (eggs)	—	44.4% (eggs and larvae)
April 13, 2000	**na**	**x (eggs)**	**na**
April 18, 2000	x (larvae)	—	55.6% (eggs and larvae)
May 16, 2000	—	—	70.3% (eggs and larvae)

Note. "x" = presence, "—" = absence and life cycle stages given in parentheses; results in **bold** are not from routine surveys. Tree infestation proportions for other waxy half-siblings are given as percentages.

surveys (Table 1). During an unscheduled survey, a single egg clutch was observed on the glossy phenotype tree (Table 1). Eggs, larvae, and moths were commonly recorded on the other waxy half-siblings.

Oviposition Choice Assays

Assay 1: Waxy Vs. Glossy Phenotypes (New and Old Leaves). More eggs were laid upon waxy (834.2 ± 96.2 eggs) than on glossy leaves (254.8 ± 66.0 eggs; ANCOVA, $P = 0.002$, $N = 11$ cages with three females per cage). The total areas of leaves on waxy or glossy branchlets in each cage (i.e., the covariate) were not significantly different ($P = 0.48$). We did not record the age of leaves where eggs were laid. Assays 2 and 3 were intended to investigate more fully the preferences of females for leaves of different ages.

Assay 2: Old Vs. New Leaves (Different Phenotypes). The trend observed in assay 1 could be modified if the ages of leaves displayed on branchlets were altered. For example, female moths laid more eggs on glossy new leaves than on waxy old leaves (assay 2i; Figure 1). This preference was exhibited even though total leaf area of waxy old leaves displayed to them was greater than that of the glossy new leaves displayed (i.e., the covariate was statistically significant, $P = 0.02$). Female moths laid more eggs on waxy new leaves than on glossy old leaves (assay 2ii; Figure 1). The difference between the total areas of waxy new or glossy old leaves was not significantly different ($P = 0.13$).

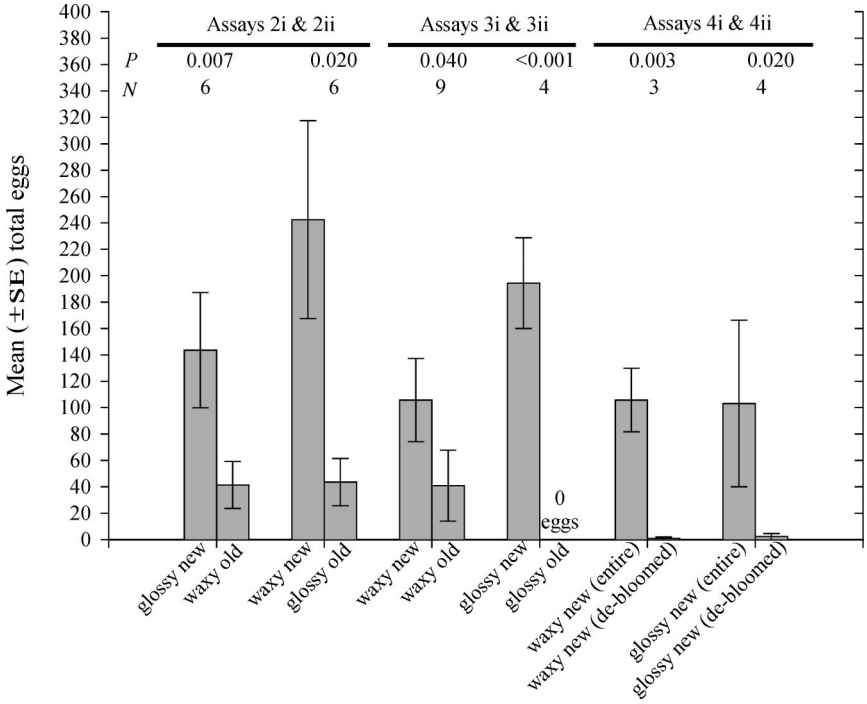

FIG. 1. Results of binary oviposition choice assays (2–4) with individual *M. privata* females (results of assay 1 detailed in the text). The probabilities from ANCOVA used to compare the numbers of eggs laid on each of the two leaf types per assay are shown in the second row at the top of the figure; the third row shows the number of cages used per assay. "Entire" = epicuticular wax layer undisturbed versus "de-bloomed" = epicuticular wax layer removed by gentle abrasion. Both de-bloomed treatments had eggs on only one branchlet, therefore, standard errors are the same as the means.

Assay 3: Old Vs. New Leaves (Same Phenotype). The mean number of eggs laid on waxy new leaves was greater than the number of eggs laid on waxy old leaves (assay 3i; Figure 1). Note that females still laid considerable numbers of eggs on waxy old leaves. The covariate was not statistically significant ($P = 0.90$). Females laid all their eggs on glossy new leaves and none on glossy old leaves (assay 3ii; Figure 1). The covariate was not statistically significant ($P = 0.65$).

Assay 4: Entire Wax Layer Vs. De-Bloomed Leaves (Same Phenotype). Females preferred to lay on leaves with entire wax layers rather than on de-bloomed leaves. This was true for both waxy new and glossy new leaves (assays 4i and ii; Figure 1). For both of these sets of assays, the covariate was not statistically significant (assays with waxy new leaves, $P = 0.13$, and assays with glossy new leaves, $P = 0.46$).

These assays were difficult to perform, and more assays were conducted than presented here. This is because the process of de-blooming by physical abrasion of the wax layer markedly increased the susceptibility of new leaves to dehydration. Once leaves dehydrate, females are deterred from laying on them.

Larval Survival and Development

All larvae reared on waxy old leaves were prepupae 38 after the assay was begun (73 after the assay was begun, the last larva reared on glossy new leaves reached the prepupal stage). The slopes of the survival curves for the groups of larvae reared on all four leaf types could only be compared up to day 38. There were no statistically significant differences in larval survival according to the type or the age of the leaves on which they were reared ($P = 0.30$). By the time all larvae were prepupae, survival varied between 72.2% (glossy new), 78.9% (glossy old), 93.8% (waxy new), and 100% (waxy old).

Larvae reared on waxy leaves took less time to pupate than did larvae reared on glossy leaves [Figure 2; Host (waxy or glossy), $F_{1,52} = 542.5$, $P < 0.001$].

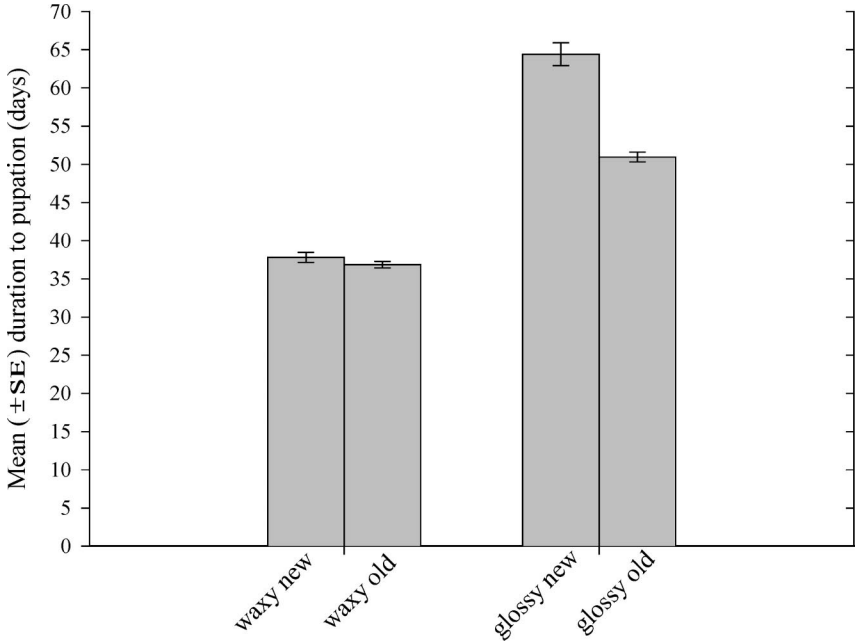

FIG. 2. Developmental durations of *M. privata* larvae reared on waxy new ($N = 15$ larvae), waxy old ($N = 13$), glossy new ($N = 13$), or glossy old ($N = 15$) leaves. Results of statistical analyses are given in the text.

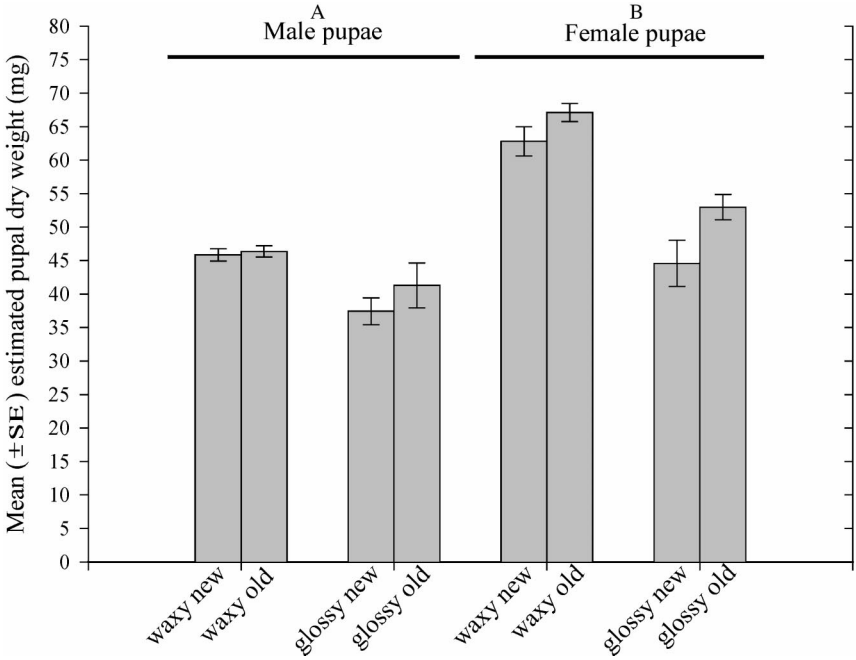

FIG. 3. Estimated dry weights of *M. privata* pupae reared on waxy new, waxy old, glossy new, or glossy old leaves. For male pupae *N*s for waxy new, waxy old, glossy new, or glossy old = 9, 6, 7, and 5 pupae, respectively, and for female pupae *N*s for waxy new, waxy old, glossy new, or glossy old = 6, 7, 5, and 10 pupae, respectively. Results of statistical analyses are given in the text.

Larvae reared on new leaves of either type were likely to take longer to pupate than those reared on old leaves [Leaf age (new or old), $F_{1,52} = 68.1$, $P < 0.001$]. This difference was most noticeable for larvae reared on glossy leaves, as reflected by a significant interaction term for Host × Leaf age ($F_{1,52} = 51.2$, $P < 0.001$).

Pupal water contents were as follows: males from waxy leaves 77.5%, females from waxy leaves 74.2%, males from glossy leaves 77.2%, and females from glossy leaves 74.7%. There was no effect of Leaf age (new or old) on male pupal weights ($F_{1,24} = 1.3$, $P = 0.27$), but the significant Host (waxy or glossy) effect ($F_{1,24} = 16.0$, $P = 0.001$) demonstrated that male pupae that had been fed waxy new leaves as larvae had dry weights greater than male pupae that had been fed glossy new leaves (Figure 3). Female pupae that had been fed waxy leaves as larvae had dry weights greater than female pupae that had been fed glossy leaves [Host (waxy or glossy), $F_{1,25} = 52.5$, $P < 0.001$] (Figure 3). This was true for both old and new leaves [Leaf age (new or old), $F_{1,25} = 8.2$, $P = 0.008$].

TABLE 2. SUMMARY OF FOLIAR MONOTERPENES OF NEW WAXY AND GLOSSY LEAVES

Monoterpene (% of total ion current)	Waxy leaves	Glossy leaves
1,8-Cineole	66.6	70.6
α-Pinene	20.6	19.6
Limonene	5.7	3.8
α-Terpineol	3.3	2.2
β-Pinene	0.8	0.6
p-Cymene	0.6	1.6
trans-Pinocarveol	0.5	0.3
Pinocarvone	0.5	0.4
Terpinene-4-ol	0.4	0.2
α-Campholene aldehyde	0.1	0.1
γ-Terpinene	0.1	0.09
Myrcene	0.1	0.04
Terpinolene	—	—

Note. $N = 1$ extract each (made from 4 leaves).

Composition of Monoterpenes

A total of 12 monoterpenes were identified from cold solvent extracts of leaves from the two trees (Table 2). These monoterpenes were present in extracts from each tree. Using the hot solvent extraction technique, we estimated the total oil content to be 45.7 ± 4.8 and 35.0 ± 9.0 mg g^{-1} dry weight for waxy and glossy leaves, respectively ($N = 2$ extracts from one leaf of each phenotype). These quantities were not significantly different ($P = 0.40$).

Composition of Epicuticular Waxes

The survey of wax components revealed that the waxy and glossy leaves differed considerably. In particular, waxy leaves were characterized by the presence of wax esters, e.g., C_{20}–C_{30} benzyl esters and C_{20}–C_{26} phenylethyl esters (Table 3). Similar esters were typically present in trace amounts in the waxes from glossy leaves. In contrast, the glossy leaves were characterized by the presence of at least four unknown compounds, possibly different alkyl benzoates. The waxes from both waxy and glossy leaves are rich in C_{31}–C_{35} β-diketones (Table 3).

Quantification of the relative amounts of a suite of eight of these wax components revealed how the two phenotypes differ, as well as how some of these compounds change in abundance as leaves age (Table 4). Waxy leaves had richer epicuticular wax profiles than glossy leaves, and new leaves were richer than old leaves. Comparing waxy new and waxy old leaves revealed statistically significant differences in the amounts of methyl eicosanoate, benzyl eicosanoate, benzyl octacosanoate, phenylethyl eicosanoate, *n*-hentriacontan-14,16-dione, and

TABLE 3. SURVEY OF ALL EPICUTICULAR WAX COMPONENTS FROM NEW WAXY AND GLOSSY LEAVES

Component (% of total ion current)	Waxy leaves	Glossy leaves	Abbreviated component name
Methyl eicosanoate	0.7	0.2	
Ethyl eicosanoate	0.9	—	
Benzyl eicosanoate	2.2	—	C_{20} benzyl ester
Benzyl heneicosanoate	0.2	—	C_{21} benzyl ester
Benzyl docosanoate	1.0	—	C_{22} benzyl ester
Benzyl tetracosanoate	0.4	—	C_{24} benzyl ester
Benzyl hexacosanaote	0.3	—	C_{26} benzyl ester
Benzyl octacosanoate	1.4	Trace	C_{28} benzyl ester
Benzyl triacontanoate	0.4	—	C_{30} benzyl ester
n-Nonacosane	0.5	1.1	
Phenylethyl eicosanoate	1.0	—	C_{20} phenylethyl ester
Phenylethyl heneicosanoate	Trace	—	C_{21} phenylethyl ester
Phenylethyl docosanoate	0.2	—	C_{22} phenylethyl ester
Phenylethyl tetracosanoate	Trace	—	C_{24} phenylethyl ester
Phenylethyl hexacosanaote	Trace	—	C_{26} phenylethyl ester
2-nonyl octadecanoate	Trace	—	
2-nonyl eicosanoate	Trace	—	
2-nonyl docosanoate	0.3	—	
2-nonyl tetracosanoate	0.3	—	
2-undecyl octadecanoate	0.6	—	
2-undecyl eicosanoate	2.0	—	
2-undecyl docosanoate	1.4	—	
2-undecyl tetracosanoate	1.3	—	
Desmethyl eucalyptin	1.4	—	
Eucalyptin	7.8	Trace	
n-Octacosanal	0.4	1.3	
n-Octacosan-1-ol	0.8	4.8	
n-Triacontanal	1.0	Trace	
n-Triacontan-1ol	2.2	Trace	
Unknown 1	—	1.0	An alkyl benzoate?
Unknown 2	—	4.3	An alkyl benzoate?
Unknown 3	—	5.0	An alkyl benzoate?
Unknown 4	—	1.0	An alkyl benzoate?
***n*-Hentriacontan-14,16-dione**	3.9	2.4	$C_{31}\beta$-diketone
***n*-Tritriacontan-16,18-dione**	55.0	76.9	$C_{33}\beta$-diketone
***n*-Pentatriacontan-16,18-dione**	6.5	2.0	$C_{35}\beta$-diketone

Note. $N = 1$ extract each (made from 2 g of fresh 1 cm^2 leaf discs). Components cited in **bold** are referred to in Table 4.

n-pentatriacontan-16,18-dione (i.e., six out of eight compounds). Wax extracts from waxy old leaves had more benzyl octacosanoate, eucalyptin, and β-diketones than did the wax extracts from glossy new or glossy old leaves (Table 4).

TABLE 4. WAX COMPONENTS AND THEIR AMOUNTS ON WAXY AND GLOSSY LEAVES

Trait	Waxy leaves		Glossy leaves	
	New leaves	Old leaves	New leaves	Old leaves
Wax components (μg tetradecane equiv. mm^{-2})				
Methyl eicosanoate				
M	0.12_a	0.002_b	0.002_b	0_b
SE	0.01	0.0005	0.0002	—
N	8	9	8	6
Benzyl eicosanoate				
M	0.57_a	0.019_b	0_b	0_b
SE	0.05	0.004	—	—
N	8	9	8	6
Benzyl octacosanoate				
M	0.11_a	0.036_b	0.007_c	0_c
SE	0.005	0.007	0.001	—
N	8	9	8	6
Phenylethyl eicosanoate				
M	0.10_a	0.007_b	0.001_b	0_b
SE	0.005	0.002	0.0002	—
N	8	9	8	6
Eucalyptin				
M	0.25_a	0.28_a	0.025_b	0.037_b
SE	0.02	0.03	0.007	0.005
N	8	9	8	6
n-Hentriacontan-14,16-dione				
M	0.97_a	0.28_b	0.06_b	0_b
SE	0.14	0.04	0.006	0.002
N	8	9	8	6
n-Tritriacontan-16,18-dione				
M	9.79_a	8.34_a	1.38_b	0.49_b
SE	1.06	0.68	0.26	0.06
N	8	9	8	6
n-Pentatriacontan-16,18-dione				
M	0.76_a	0.37_b	0.05_c	0.01_c
SE	0.10	0.06	0.01	0.001
N	8	9	8	6
Waxes per unit area (μg mm^{-2})				
M	4.0_a	3.1_{ab}	2.4_{bc}	1.8_c
SE	0.1	0.1	0.5	0.1
N	12	11	11	12

Note. Means in the same row with the same subscript letter are not significantly different from each other.

Only eucalyptin did not decline in amount as the leaves of either type aged. If anything, there may even have been a slight increase in the amount of eucalyptin on old leaves compared to new leaves (Table 4). Waxy new leaves had more waxes

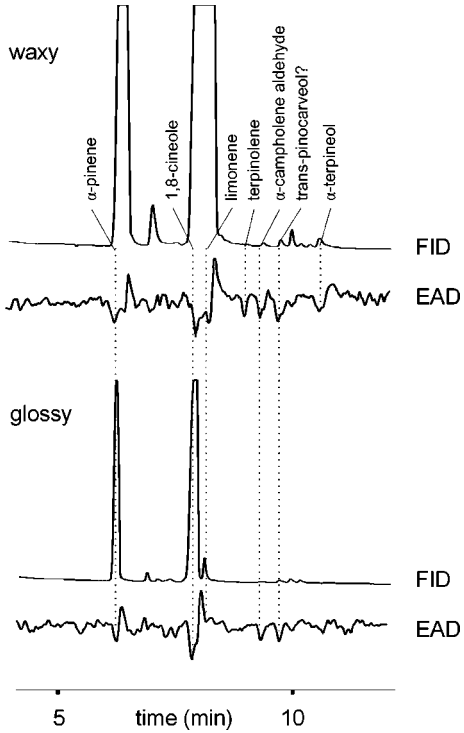

FIG. 4. Recordings of FID and EAD traces of responses of female *M. privata* antennae to headspace extracts of foliar monoterpenes from branchlets of the waxy and glossy phenotype trees. Traces represent one run of each sample using two antennae from a single individual. EAD active monoterpenes have been named.

per unit area than did waxy old leaves, which had approximately the same amount of wax per unit area as glossy new leaves, which had more wax per unit area than glossy old leaves (Table 4).

Electrophysiological Studies

Antennal Responses to Foliar Monoterpene Odors and GC-EAD. The antennae of females did not respond to all monoterpenes. Of the 12 monoterpenes, we were able to identify (Table 2) only 7 from the waxy tree and 5 from the glossy tree elicited responses in antennae from female moths (Figure 4). The main active compounds from waxy branchlets were 1,8-cineole, α-pinene, limonene, and α-terpineol (Figure 4). In addition, we recorded antennal responses to comparatively minor components, including α-campholene aldehyde and terpinolene. Responses to 1,8-cineole, α-pinene, and limonene were also observed for

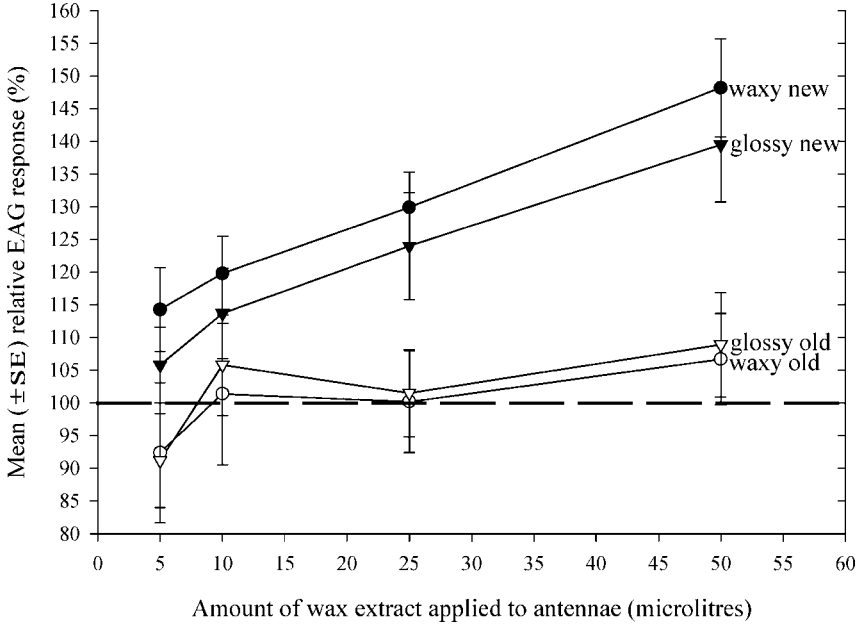

FIG. 5. EAG responses of female *M. privata* antennae to epicuticular waxes from new and old leaves from the waxy and glossy phenotype trees. Dotted bar at 100% indicates response of antennae to nonspecific stimuli (e.g., responses of mechano-receptors to air movement, see Methods and Materials). $N = 9$ females for extracts from waxy new leaves and $N = 7$ females for all other extracts. Results of statistical analyses are given in the text.

extracts from glossy branchlets, and we also recorded a response to α-campholene aldehyde (Figure 4).

Antennal Responses to Epicuticular Wax Odors and EAG. We could not record antennal responses to epicuticular waxes using GC-EAD. When female antennae were stimulated with the wax odors of new leaves, a positive dose-dependent response was recorded (Figure 5). This was true for both waxy and glossy phenotypes. In contrast, positive dose-dependent responses were not recorded when female antennae were exposed to wax odors of extracts from old leaves of either tree. The antennal responses to the odors of waxy new leaves were not significantly different from those of extracts of glossy new leaves. The magnitudes of the responses to extracts from old leaves were not significantly different according to the tree from which they were derived.

By combining data for responses to waxy and glossy waxes, we found that responses to 25 and 50 μl of extract from new leaves were larger than responses to either 5 or 10 μl of extract from new leaves or any of the responses to extracts from old leaves (new vs. old at 25 μl, $P = 0.007$, and similarly at 50 μl, $P = 0.001$). Female antennae responded differently to wax odors from new leaves than they

did to those from old leaves, and the responses were most noticeable at 25 μl or greater amount of extract (Figure 5).

In pair-wise comparisons, we found differences between the magnitudes of the responses to 25 ($P = 0.004$) and 50 ($P = 0.001$) μl of waxy new vs. waxy old extract. Likewise, 25 ($P = 0.03$) and 50 ($P = 0.01$) μl of glossy new vs. glossy old extract differed from each other. No other comparisons were statistically significant.

DISCUSSION

The glossy putative hybrid attracted our attention because fewer *M. privata* were observed on it compared to the number of occurrences of the insect on other waxy half-siblings, such as the half-sibling we selected for use in our studies. This evidence suggested that some leaf characteristics of this tree were not as attractive (more than one leaf trails makes the hybrid unattractive to *M. privata*) to *M. privata* as those of its half-siblings. Among the most prominent differences was the significantly lower amount of wax. The size and color of glossy leaves also differed from those of waxy leaves, but the possible influence of these characteristics was not addressed (leaf size was used as the covariate in our analyses of data from the oviposition choice assays). Extracts of foliar monoterpenes from the glossy and waxy trees indicated that their leaves had the same components. Furthermore, the total oil contents of the two were not different. Headspace extracts of volatiles from each tree confirmed these similarities, and the relatively low concentration of certain odors from glossy leaves meant that we did not record antennal responses to them, e.g., α-terpineol. This is the first time that the antennae of a native Australian insect have been shown to respond to the monoterpene odors of its eucalypt host. Städler (1974) and Leather (1987) have suggested that foliar monoterpenes are used by some moth species to assist with discrimination between hosts. It could be that female *M. privata* use the differences in monoterpene composition of the two trees to differentiate between them.

SEM supported differences apparent to the naked eye concerning the quantity (but not the structure) of the waxes on the undersides of leaves. To date, epicuticular waxes have only been suggested to act as a physical defense against eucalypt-feeding insects, in particular against leaf beetles (Edwards, 1982; Edwards and Wanjura, 1990). Steinbauer (2002) observed that female *M. privata* are able to cling onto waxy juvenile foliage and actually prefer to lay eggs on the waxier undersides of leaves. This indicates that epicuticular waxes do not prevent moths from adhering to the surfaces of leaves. Jones et al. (2002) reported that some genotypes of *E. globulus*, whose waxes contained higher amounts of C_{23}–C_{26} benzyl and phenylethyl esters, suffered less defoliation by *M. privata*. They reported that benzyl tetracosanoate, in particular, was consistently correlated with higher resistance to defoliation. These authors did not demonstrate a mechanism through which reduced defoliation could arise, but changes in oviposition in response to

altered wax composition are a possibility because epicuticular waxes can act as oviposition stimulants (Udayagiri and Mason, 1997; Morris et al., 2000; Müller and Hilker, 2001) or deterrents (Justus et al., 2000). The nonpreference of *M. privata* for glossy over waxy leaves in the field and in the laboratory suggests that waxes act as oviposition stimulants for *M. privata*.

If we accept that epicuticular waxes are oviposition stimulants for *M. privata*, the next issue to arise is whether it is the amount, composition, or both that influences the responses of females. Waxy new leaves had the greatest quantity of waxes per unit of leaf area, while glossy old leaves had the lowest. The quantities of almost all of the components declined as leaves aged, irrespective of the tree from which they came (the possible exception was eucalyptin). These data suggest that it is not possible, at this stage, to state categorically that it is the amount or composition alone that influences a female's oviposition behavior. Also, it should not be ruled out that waxes may influence the emission of monoterpene odors and could confound separating the influences of different cues. Further, Morris et al. (2000) reported that specific components of the epicuticular waxes of host leaves stimulated oviposition by the hessian fly. Because females will oviposit on leaves of the glossy tree, albeit to a lesser extent than on leaves of the waxy tree, it seems likely that one or more of the wax components on this tree is the oviposition cue for *M. privata*. Moreover, oviposition was considerably reduced when leaves were de-bloomed. For example, when new leaves were de-bloomed of their waxes, they were less preferred to leaves of the same age from the same host. Had leaves on pairs of branchlets in the de-blooming assays been entire, we would have expected both to have been equally oviposited upon because new leaves were always preferred by females. In effect, we rendered new leaves equivalent to old leaves by abrading their epicuticular wax layers. Notice, however, that if C_{23}–C_{26} benzyl and phenylethyl esters were responsible for deterring oviposition by *M. privata* (as could be inferred from Jones et al., 2002), then the leaves of the glossy tree should be preferred to those of the waxy tree because they are typically low or devoid of these components. If the waxes on the leaves of the glossy tree include the biologically active components, it narrows the field of compounds that need to be investigated further before a definitive identification can be given.

Our assays demonstrate that leaf age is assessed by female *M. privata* by using epicuticular waxes as an indicator or proxy. Our EAG studies provide further support for the suggestion that waxes are used as an indicator of leaf age. We are not aware of any study that has shown that epicuticular waxes are used by ovipositing insects to assess the quality of different leaves on the same host.

M. privata females prefer waxy leaves to glossy leaves. This was not reflected in poorer survival of larvae on the latter tree's leaves. Leaves from both trees, whether new or old, enabled more than 70% of larvae to reach pupation, i.e., glossy leaves did not harm larvae even though females avoided them. Although estimated survival rates showed some variation, differences were not statistically significant. It should be noted that the leaves we studied had SLWs lower than 0.20 mg mm^{-2}

that appears to be a threshold of leaf toughness associated with reduced larval survival (Steinbauer, 2002). Although survival did not differ, larvae reared on glossy leaves took longer to reach pupation than those reared on waxy leaves. Furthermore, pupae reared on glossy leaves generally weighed less than those reared on waxy leaves. Consequently, both the time to pupation and the weight attained by pupae indicate that there are fitness benefits to *M. privata* by avoiding the glossy tree when ovipositing. We assume that the developmental advantages of feeding upon waxy leaves could have consequences for the fitness of *M. privata* because rapid larval development decreases the chances of individuals being killed by natural enemies and reduces the time before individuals can reproduce (see Benrey and Denno, 1997; Hunter and McNeil, 1997). In addition, lower female body weight can reduce fecundity (Haukioja and Neuvonen, 1985; Tammaru et al., 1996). The disadvantages to *M. privata* of depositing eggs on leaves of the glossy tree demonstrate the importance of being able to recognize preferred hosts in habitats where there are many other closely related plant species. We suggest that that female *M. privata* use monoterpenes and possibly also waxes to assist with host plant location and selection.

Our findings demonstrate the importance of epicuticular waxes to host plant assessment, in particular the assessment of leaf age by ovipositing *M. privata* females. However, they do not present an entirely satisfactory explanation for why females prefer new leaves. For example, larvae reared on new leaves had increased developmental times and reduced body weights. If new leaves do not benefit the development of larvae, are there other reasons for selecting them that are not related to growth? One nonnutritional reason for selecting new leaves may be the necessity for late instars to bend and silk together leaves into a shelter in which they hide during the day (pictured in Steinbauer et al., 2001). The role of these leaf shelters is not known, but all larvae, either individually or in groups, make them. It has been shown that the adults of some other insects make choices about the locations their offspring will develop in that are not related to host quality. For example, female *Yponomeuta mahalebella* Latr. (Lepidoptera: Yponomeutidae) deposit their eggs in locations on individual hosts where their larvae will later be able to form leaf shelters that will experience higher insolation (Alonso, 1997). The increased temperatures inside leaf shelters that experience the most insolation benefit larval survival and development. Whether *M. privata* derives similar advantage from leaf shelters or whether there are yet other important aspects of its host utilization presents interesting avenues for future research (see Danks, 2002; Sipura et al., 2002).

The utilization of changes in the composition of epicuticular waxes to differentiate among leaves of different ages could be widespread in insects native to regions where they must utilize poor-quality host plants whose good-quality foliage is ephemeral. Furthermore, our suggestion that monoterpenes as well as waxes may be used to discriminate among hosts seems relevant to insects that utilize evergreen "aromatic" and glaucous plant species (e.g., conifers, see Ross et al., 1995), especially those in habitats where the array of related plant species is high.

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INDUCED DEFENSIVE RESPONSE OF MYRTLE OAK TO FOLIAR INSECT HERBIVORY IN AMBIENT AND ELEVATED CO_2

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Abstract—The rising level of atmospheric CO_2 has stimulated several recent studies attempting to predict the effects of increased CO_2 on ecological communities. However, most of these studies have been conducted in the benign conditions of the laboratory and in the absence of herbivores. In the current study, we utilized large octagonal chambers, which enclosed portions of an intact scrub-oak community to investigate the interactive effects of CO_2 and insect herbivory on myrtle oak, *Quercus myrtifolia.* Specifically, we assessed the effects of ambient and elevated CO_2 ($2\times$ current concentrations) on percent foliar nitrogen, C:N ratio, total relative foliar tannin content, and the presence of leaf damage caused by leaf mining and leaf chewing insects that feed on myrtle oak. Total foliar N declined and C:N ratios increased significantly in oaks in elevated CO_2 chambers. The percentages of leaves damaged by either leafminers or leaf chewers tended to be lower in elevated compared to ambient chambers, but they co-occurred on leaves less than expected, regardless of CO_2 treatment. Leaves that had been either mined or chewed exhibited a similar wounding or defensive response; they had an average of 25 and 21% higher protein binding ability, which is correlated with tannin concentration, compared to nondamaged control leaves, respectively. While the protein-binding ability (expressed as total percent tannin) of leaves from elevated CO_2 was slightly higher than from leaves grown in ambient chambers, this difference was not significant.

Key Words—Induced defense, tannins, C:N ratio, leafminers, *Quercus myrtifolia*, elevated CO_2.

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INTRODUCTION

Concentration of atmospheric CO_2 is currently increasing at a rate of approximately 1.5 μl/1 yr largely owing to increased use of fossil fuels and large scale deforestation, and is expected to double within the next century from its current level of 360 to 700 μl/l (Wigley and Raper, 1992; Keeling et al., 1995; Houghton et al., 1996). Plants in general, and trees in particular, account for an estimated 70% of all CO_2 fixed by terrestrial ecosystems, and forests are expected to mitigate some of the effects of rising CO_2 by sequestering carbon in their tissues and in the soil litter layer (Oren et al., 2001; Schlesinger and Lichter, 2001). Alterations in atmospheric CO_2 concentration may affect global temperatures (Flower, 1999; Crowley and Berner, 2001; Sandalow and Bowles, 2001), plant quality, and rates of herbivory (Mansfield et al., 1999; Stiling et al., 1999; Villalba et al., 2002; Heagle, 2003), plant biomass (Oren et al., 2001; Heijmans et al., 2002) as well as ecosystem community composition (Tangley, 2001; He et al., 2002; Heijmans et al., 2002). Although photosynthetic rates and plant growth are expected to increase in elevated CO_2, recent studies suggest that these effects are dependent upon soil fertility as well as temperature. Thus, the ability of forests to absorb increasing amounts of carbon may be limited (Li et al., 1999; Oren et al., 2001; Schlesinger and Lichter, 2001). For instance, Clark et al. (2003) recently reported that the growth of six, old-growth tropical rain forest canopy tree species was negatively correlated with temperature (possibly due to increased plant respiration at higher temperatures). As a result, higher temperatures associated with increasing atmospheric CO_2 may feed back to the plant community and cause a decrease in the net primary production of forests.

Forest ecosystems also support a wealth of herbivores, and herbivory can also affect forest production and carbon sequestration. Elevated CO_2 may affect the levels of defensive chemicals (i.e., secondary compounds) used by trees to protect themselves from herbivores (Agrell et al., 2000; Hartley et al., 2000; Lindroth et al., 2001). Bryant et al. (1983) suggested that slow-growing boreal forest trees should utilize carbon-based (C-based) secondary compounds because photosynthesis, which plants use to fix CO_2 into plant biomass, is less limiting to the plant than are soil-derived nutrients; primarily nitrogen (carbon/nutrient balance hypothesis or C/N hypothesis). Additionally, increased concentrations of atmospheric CO_2 may result in increased levels of C-fixation and elevated production of C-based secondary compounds.

Tannins are typically divided into two major classes: condensed and hydrolyzable (Bernays and Chapman, 1994). Condensed tannins or proanthocyanidins are polymers of flavanols, while hydrolyzable tannins are phenolic acid esters of polyols (typically D-glucose) (Bernays and Chapman, 1994; Kraus et al., 2003). Tannins are found in most classes of vascular plants; oaks, *Quercus* spp., are well-known producers (Swain, 1979; Harborne, 1988). Tannins cross-link proteins,

which causes them to precipitate out of solution and prevents digestive enzymes such as trypsin from effectively breaking them down; this reduces the amount of extractable nitrogen from the plant (Feeny 1969; Bernays and Chapman 1994, but see Mole and Waterman, 1985). To date, research on the effects of elevated CO_2 on the production of carbon-based compounds has produced mixed results with some studies showing increases in tannin levels (Lindroth et al., 1993, 1995, 2001; Agrell et al., 2000) and others showing no effect (Fajer et al., 1991; Berenbaum and Seigler, 1992; Williams et al., 1997; Hartley et al., 2000). Lindroth et al. (1995) found that condensed tannin concentrations in paper birch (*Betula papyrifera*) doubled when trees were grown in elevated CO_2, while Fajer et al. (1991) reported that neither iridoid glycoside nor fiber concentrations in *Plantago lanceolata* were affected by CO_2 levels. Additionally, most studies that have examined the effects of elevated CO_2 on the production of tannins have been conducted under nonstressful laboratory conditions in which water and nutrient availability were not limiting and herbivores were absent. However, in most terrestrial ecosystems, many leaves suffer damage from insect herbivores and soil fertility is limited. Tannin levels have been shown to increase in response to leaf damage, yet most studies have assayed tannin concentrations in elevated CO_2 using only nondamaged leaves (Drury et al., 1998; Roth and Lindroth, 1994; Lindroth et al., 1995, 2001; Roth et al., 1998; McDonlald et al., 1999; Hartley et al., 2000), despite the fact that 30–50% of leaves may be damaged in mature forests (and up to 70% of leaves on some trees are damaged in our study system, *personal observation*). In the current study, we assessed the effects of elevated atmospheric CO_2 on the levels of tannin produced by leaves that had been damaged by insect herbivores and nondamaged (control) leaves of myrtle oak, *Quercus myrtifolia*.

METHODS AND MATERIALS

Study Site. The study was conducted in conjunction with the long-term elevated CO_2 enrichment experiment being run by the Smithsonian Institution and the National Aeronautics and Space Administration (NASA), which is located on a 2-ha site at the Kennedy Space Center near Titusville, Florida. Because this investigation was conducted in conjunction with the NASA elevated CO_2 project, which is a unique experimental system involving multiple investigators, we were limited in our ability to manipulate and sample trees. As a result, data are correlative and the results suggestive, but they warrant further investigation. The site is representative of the low-nutrient, mature scrub habitat that is characteristic of the area. This fire-maintained ecosystem is dominated by two scrub oak species, sand live oak (*Quercus geminata*) and myrtle oak (*Q. myrtifolia*), which account for $>$90% of the biomass at the site (Stiling et al., 1999). During winter 1996, prior to initiation of the experiment, the area was burned and all vegetation was cut back to ground level.

In the spring of 1996, sixteen (3.6 m diam $\times$ 3.4 m height) open-top, octagonal chambers were constructed *in situ* over the native scrub oak community using polyvinylchoride frame panels covered with a clear polyester film (for a detailed description of the site, see Li et al., 1999 or Stiling et al., 1999). Chambers, which were open at the top, allowed gases to vent, natural rainfall to reach the vegetation inside, and enabled insects to migrate in and out of chambers. The sixteen chambers were paired into eight groups; one member (control) of each pair received ambient levels of atmospheric CO_2 ($\approx$350 μl/l), while the other (elevated) received approximately twice current CO_2 levels ($\approx$700 μl/l) since 1996. To minimize fluctuations in CO_2 concentrations, leaves were censused from the outside of the chambers when possible. If a chamber had to be entered to census leaves, the researcher stood on designated footholds to minimize disturbance to the vegetation, and the chamber door was closed during sampling to reduce gas loss. Although temperatures inside chambers are slightly higher than nonchambered plots, there was no temperature difference between ambient and elevated chambers (Li et al., 1999).

Leafminer Abundance and Leaf Damage. The study focused on the abundance of a guild of lepidopteran leafminers that attack the leaves of myrtle oak, *Q. myrtifolia*. Leafminers are especially useful for herbivory studies because female moths lay eggs on the surface of the leaf; after hatching, the larvae burrow into the tissue between the upper and lower epidermis. This active (feeding) larval stage is confined to the leaf mine and, as the larva develops and increases in size, the mine (area consumed by the leafminer) shows concomitant increase in size.

In March 2001 (just after leaf flush), 100 leaves from a randomly selected myrtle oak tree in each chamber were sequentially numbered with an indelible pen (800 leaves per treatment, 1600 total). Each leaf was scored monthly from March–December for the presence of leafminers or damage caused by chewing insects until leaf abscission. Although myrtle oak is considered evergreen, it typically abscises most leaves in January–February and quickly produces new foliage in March at our study site. After 9 months, differences in mean percent of leaves with leafminers and chewed leaves on trees from ambient and elevated chambers were assessed using paired *t*-tests. Co-occurrence of leafminers and leaf chewers (as indicated by chewing damage) was assessed using a χ^2 contingency table.

Chemical Analysis of Leaves. Two nondamaged leaves were collected in December 1999 from each chamber for chemical analyses during December 1999, which is at the end of the growing season (just prior to leaf abscission). Leaves were randomly selected from the same branches of myrtle oak that had been sampled for insect densities. After collection, leaves were oven dried at 65°C for 5 days, ground into a fine powder using a Wiley Mill, and then used to determine the concentrations of carbon and nitrogen. Nitrogen and carbon concentrations, which were expressed as percent dry mass using a Perkin–Elmer CHN analyzer

(see Rossi and Strong, 1991, for details), were used to calculate C:N ratios for the treatment groups.

We also collected 3–5 leaves from each chamber to determine mean total tannin concentrations for the following leaf classes: (1) leaves that had no damage (control); (2) leaves that had a leaf mine (mined); and (3) leaves that had damage caused by chewing insects such as caterpillars and grasshoppers (chewed). Total (protein-binding) foliar tannin concentration was determined by using the radial diffusion assay developed by Hagerman (1987, 1988). We used total tannin concentration, rather than isolating condensed and hydrolyzable tannins, because this technique provides an assay of the total binding ability of tannin in the leaves, which has a biologically meaningful value. For each leaf, two replicate samples were assayed for protein-binding ability, and the mean value of the replicates was used to calculate total tannin concentration as a percent of dry leaf mass. For tannin analysis, 50 mg of dried leaf sample were placed into 250 μl of a solvent solution containing a 50% aqueous acetone solution, which has been shown to be the most effective solvent for extracting tannins (Cork and Krockenberger, 1991). The agar medium used for the tannin assay contained bovine serum albumin (BSA, Sigma Chemical Company, St. Louis, MO), and as tannins diffuse into the agar from the sample well they cross-link with the BSA, which creates a bright white precipitation ring. The diameter squared of this ring is linearly correlated with the amount of tannin in the sample. Plates were placed into an incubator (30°C) for 72 hr and then precipitation ring diameters were measured under a drafting light/magnifier (10×) using digital dial calipers (accurate to 0.01 mm).

Since the commercial tannin standard used for sample comparison in the current study was not isolated from the same oak species (D. Bowersox, personal communication, ICN Biomedicals, Aurora, IL), our results represent *relative* total tannin concentrations rather than absolute values. Linear regression analysis was used to generate a standard line describing the relationship, which was ring diameter (cm^2) = 9.41 (tannin concentration) +0.225; $r^2 = 0.992$; $P < 0.001$, accurate to 0.025 mg. For each chamber, mean total tannin for each leaf category was then converted into a percent of dry leaf mass and analyzed using a split–plot analysis of variance (ANOVA) in which CO_2 treatment (ambient or elevated) was the main effect and leaf condition (control, chewed, or mined) was nested within CO_2 treatment.

RESULTS

Leafminer Abundance and Leaf Damage. Although leafminer abundance was 44% lower on myrtle oak in elevated chambers compared to ambient ones, this difference was only marginally significant ($t = 1.80$; $df = 14$; $P = 0.096$).

TABLE 1. EFFECTS OF ATMOSPHERIC CO_2 ON PLANT CHEMISTRY AND INSECT DAMAGE IN MYRTLE OAK[a]

Treatment	Leaf condition	% *N*	C:N	Total tannin[b] (% dry mass)	% Leaves mined	% Leaves chewed
Ambient (350 μl/l)	Control	1.22 ± .09a	33 ± 2a	5.8 ± 0.4a	—	—
	Mined	—	—	7.2 ± 0.3b	5.4 ± 1.1a¥	—
	Chewed	—	—	7.1 ± 0.4b	—	35.6 ± 5.5a§
Elevated (700 μl/l)	Control	1.03 ± .07b	38 ± 2b	6.0 ± 0.4a	—	—
	Mined	—	—	7.5 ± 0.4b	3.0 ± 0.9b¥	—
	Chewed	—	—	7.2 ± 0.3b	—	22.6 ± 4.5b§

[a] Values are mean ± SEM. Values followed by different letters are statistically different at $\alpha = 0.05$; values followed by letters and symbols within the same column are significant at the α level indicated: ¥ $P = 0.096$; § $P = 0.072$.

[b] Values represent relative total values (see text for details).

Similarly, percent of leaves damaged by leaf chewers was 57% higher in ambient chambers compared to elevated ones ($t = 1.95$; $df = 14$; $P = 0.072$; Table 1). Approximately 31% (495 out of 1600) of the marked leaves were damaged either by leafminers, leaf chewers, or both. Because both ambient and elevated CO_2 treatments showed the same trends, the data from both treatments were pooled prior to analyses. Leafminers and leaf chewers co-occurred significantly less than expected, regardless of CO_2 treatment ($\chi^2 = 14.43$; $df = 1$; $P < 0.001$); 69 leaves had mines, 422 had chewing damage, and only 4 were both chewed and mined (no mines co-occurred).

Chemical Analysis of Leaves. CO_2 treatment had a significant effect on foliar nitrogen content; trees grown in ambient chambers had an average of 21% more nitrogen than trees grown under elevated CO_2 ($t = 2.38$; $df = 13$; $P = 0.036$). As a result, foliar C:N ratio was also affected by CO_2 level ($t = 2.72$; $df = 13$; $P = 0.018$). The C:N ratio of leaves from elevated chambers was approximately 15% higher than that in leaves from ambient chambers (Table 1). Total protein-binding foliar tannin concentrations were higher in damaged leaves, whether mined or chewed, compared to nondamaged control leaves ($F_{2,28} = 8.45$; $P = 0.001$). Leaves that had been either mined or chewed had an average of 25 and 21% more total tannin than nondamaged leaves, respectively (Table 1). Although leaves from trees in elevated CO_2 chambers were consistently higher in total tannin concentration for all three categories (mined, chewed, and nondamaged controls), the overall effect of CO_2 treatment was not significant ($F_{1,14} = 0.012$; $P = 0.916$). The interaction between CO_2 treatment and leaf condition was not significant because trees grown under both ambient and elevated CO_2 responded similarly in their production of tannins in response to leaf chewing or mining damage ($F_{2,28} = 0.164$; $P = 0.850$).

DISCUSSION

Our results were in partial agreement with the carbon/nutrient hypothesis. For instance, leaves in elevated CO_2 were significantly lower in N than ambient leaves, but their modest increase (mean = 3% higher for all three leaf categories) in total foliar tannin was not significantly different from ambient leaves. This seems somewhat surprising since Li et al. (1999) found that myrtle oak growing in elevated CO_2 chambers in this same study system exhibited a 51% increase in photosynthetic rate (i.e., carbon fixation), which resulted in a 54 and 264% increase in the accumulation of sugars and starch, respectively. Since we do not know if leaves we sampled for tannins from elevated CO_2 chambers in 1999 maintained the higher rates of photosynthesis detected in 1996, it is possible that the increase in photosynthetic rate of leaves in elevated CO_2 was transient. For instance, Oren et al. (2001) reported that the early increases in plant biomass and carbon sequestration exhibited by loblolly pines exposed to elevated CO_2 were temporary; these gains were lost after approximately 3 years of exposure to elevated CO_2. Our study, which was conducted more than 3 years after the CO_2 project had been initiated, suggests that the myrtle oak leaves in elevated chambers, while induced to respond to damage by producing elevated levels of tannins, did not respond any more strongly than leaves in ambient CO_2.

However, both the abundance of the guild of leafmining lepidopterans and damage caused by leaf chewing insects attacking myrtle oak were depressed in elevated CO_2. Previous studies have found a similar decline in the densities of other phytophagous insects such as leaf-tiers and wooly aphids in this system (Stiling et al., 1999, 2002). Although total foliar tannin concentration was not significantly higher in leaves from elevated CO_2 chambers, Stiling et al. (1999, 2003) found that leafminers in elevated chambers consume significantly (30–40%) more leaf area than those in ambient chambers. As a result, leafminers in elevated CO_2 chambers, which produce larger mines (and consume more leaf tissue), may ingest significantly more tannin over the course of their development, even though total tannin concentration may not be significantly higher in these leaves.

Our results suggest that leaf damage elicited a wounding or a defensive response in myrtle oak regardless of atmospheric CO_2 concentration. Compared to nondamaged control leaves, foliar tannin concentrations increased by more than 20% in leaves that had been damaged either by leafminers or leaf chewers. Leaf mines co-occurred on leaves that had chewing damage significantly less often than expected, which indicates that once a leaf receives damage from either mining or chewing insects, an increase in foliar tannins induced by wounding, renders the damaged leaf less attractive to subsequent leafminers or leaf chewers. Tannin concentrations affect both larval survival, development time, and food consumption rates/efficiencies in other lepidopteran herbivores (Lindroth et al., 1995; Kopper et al., 2002), and increased tannin levels in response to feeding damage have been

suggested for several plant species (Feeny and Bostock, 1968; Feeny, 1970a,b; Schultz and Baldwin, 1982). However, owing to our limited sampling ability, it is possible (although unlikely) that insect herbivores prefer high-tannin leaves or that they avoid occupied leaves to reduce competition.

More studies using seminatural systems are required to predict if plants, especially trees, will be able to remove the bulk of additional carbon from the atmosphere (as storage carbohydrates or C-based secondary compounds) as CO_2 concentrations rise. Because most forest ecosystems have nutrient-poor soils, which can limit rates of C-fixation, the ability of trees to offset expected increases in atmospheric CO_2 may be overly optimistic (Oren et al., 2001; Zurer, 2001; Clark et al., 2003).

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THE EFFECT OF SELECTED MONOTERPENOIDS ON THE CELLULAR SLIME MOLD, *Dictyostelium discoideum* NC4

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Abstract—We tested the activity of 11 main compounds identified from *Pinus* plants on the growth of *Dictyostelium discoideum* NC4. Four concentrations (1, 0.1, 0.01, 0.001 μg/μl) of each compound were tested using a disk volatilization technique following germination of *D. discoideum* NC4 spores. Photographs of *D. discoideum* NC4 fruiting bodies were taken 2 days after treatment. Fenchone (at 0.1, 0.01, and 0.001 μg/μl) and camphene (at 0.01 μg/μl) stimulated growth of *D. discoideum* NC4. (1*S*)-(−)-verbenone, (1*S*)-(−)-α-pinene, (+)-β-pinene, myrcene, (−)-menthone, (−)-bornyl acetate, (*S*)-(+)-carvone, (−)-camphene, and (*R*)-(+)-limonene inhibit its growth. All of the compounds at 1 μg/μl had a strong inhibitory effect on cell growth of *D. discoideum* NC4. Microscopic observation of the fruiting bodies matched the results of growth rate analysis. Most of the inhibitory effects were represented by changes in the shapes of the fruiting bodies. These changes include short sorophores, smaller sized sori, and sori without spores. Our results suggest that inhibition of growth is the most common effect of monoterpenoids on *D. discoideum* NC4. Nevertheless, some of them, like fenchone and camphene, seem to enhance its growth.

Key Words—*Dictyostelium discoideum* NC4, monoterpenoids, disk volatilization, inhibition, fruiting body.

INTRODUCTION

Some monoterpenes from living leaves might manage, under certain circumstances, to make their way into topsoil through a number of possible mechanisms (Harborne, 1991). These compounds could influence soil microorganisms

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in the humus layers and topsoil. Recently, it was suggested that degradation of monoterpenes in Scots pine needle litter is a slow process, and compounds could have effects on decomposer organisms for several years after needle abscission (Coûteaux et al., 1998; Kainulainen and Holopainen, 2002). Small amounts of terpenes corresponding to naturally occurring concentrations are active against a wide variety of fungi and bacteria (Misra et al., 1996; Oosterhaven et al., 1996; Helander et al., 1998).

There are varying results suggesting that monoterpenes both stimulate (Vokou and Liotiri, 1999; Schmidt et al., 2000; Vokou et al., 2002) and inhibit the growth of soil microorganisms (White, 1994; Amaral et al., 1998; Paavolainen et al., 1998). Microbial degradation of monoterpenes under anaerobic conditions has been described by Harder and Probian (1995), who suggested that anaerobic bacteria use monoterpenes as sole carbon and energy sources. Such differing results depend on the microorganisms, compounds, and concentrations examined. Monoterpenes may play a role in the control of microbial processes in environments where they are abundant, such as pine forest soils (Coûteaux et al., 1998; Paavolainen et al., 1998).

Cellular slime molds (CSM) or dictyostelids are common inhabitants of most litter-rich soils, where they usually exist as free-living amoeba-like cells, or myxamoebas. They feed on bacteria, which decompose organic material from dead plants, especially fallen leaves and dead wood. Therefore, CSMs have an important role in the ecosystem of forest soil (Feest and Madelin, 1988). *Dictyostellium discoideum* NC4 is present in pine forest floors (Cavender, 1972; Hagiwara, 1996; Hwang et al., 2000). Evaluation of the activity of monoterpenes present in pine forest floor on its growth would add to our knowledge about soil processes in terpene-rich environments. To our knowledge, the activities of monoterpenes that are present in pine forest floor on CSM have not been previously reported. The aim of this study is to investigate the effect of selected monoterpenoids on *Dictyostellium discoideum* NC4 growth during its lifespan.

METHODS AND MATERIALS

Culture Methods. We obtained *D. discoideum* NC4 from the Department of Botany at Kyoto University in Japan. *D. discoideum* NC4 amoebae were grown at 22°C on lawns of *E. coli* plated on nonnutrient 2% agar media (Hagiwara, 1998). *E. coli* was supplied as a feeding source for *D. discoideum* NC4, which was grown in nutrient agar and incubated at 37°C for 2 days.

Monoterpenes and Dilution. The composition of monoterpenes in *Pinus* plants (*P. densiflora*, *P. thunbergii*, and *P. rigida*) was studied by Kang and Kim (1997). We selected 11 monoterpenes representing the main constituents of *Pinus*,

and purchased them from Aldrich Chemical Inc. and Fluka Chemical Company. They were myrcene, (*R*)-(+)-limonene, (−)-menthone, (*S*)-(+)-carvone, (1*R*)-(−)-fenchone, α-pinene, (−)-camphene, (1*S*)-(−)-verbenone, β-pinene, geranyl acetate, and bornyl acetate. Each monoterpene was tested individually at four concentrations (1, 0.1, 0.01, 0.001 μg/μl). A 1 μl aliquot of compound was added to filter paper (Millipore hawp04700, 5 mm diam). Axenic liquid media (per liter, 14.3-g bacteriological peptone, 7.15-g yeast extract, 30.8-g D-glucose, 1.28-g $Na_2HPO_4 \cdot 12H_2O$, and 0.49-g KH_2PO_4, final pH 6.7) as indicated by Watts and Ashworth (1970) was used for dilution. The axenic solution alone does not cause inhibition or enhacement of CSM amoebae growth. The culture solution with no monoterpenoids was used as the control.

Disk Volatilization. Many researchers (Tellez et al., 2001; Thangadurai et al., 2002; Vokou et al., 2002) have tested the effect of volatile oils on microorganisms with a disk diffusion technique, measuring the zone of inhibition. However, we could not use this technique. *D. discoideum* NC4 migrate after they aggregate in myxamoebae, and no clear zone is formed. To address this, we devised the disk volatilization technique (Figure 1). Filter paper disks containing 1 μl of compound were applied to the cap of microtubes located on the upper surface of CSM.

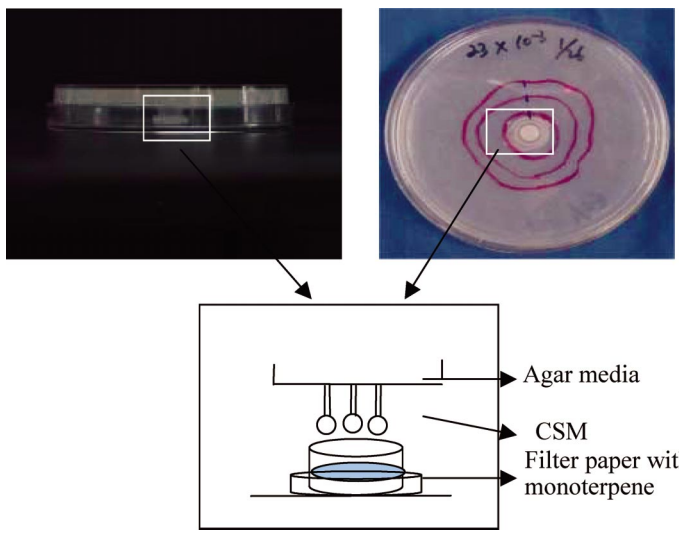

FIG. 1. Culture plate and the scheme of *D. discoideum* NC4 by the disk volatilization technique. Left photo (side view) shows 2% agar media in the upper side, the filter paper inside microtube cap is located in the lower side. Right photo (top view) shows growth area of *D. discoideum* NC4.

Each compound was tested after the germination of *D. discoideum* NC4 spores. The surfaces of 2% agar medium plates were inoculated with *D. discoideum* NC4, and then incubated at 22°C for 3 days. The growth area was measured by planimeter at 24-hr intervals. We estimated growth rates in the presence of the various compounds at each concentration examined. Inhibition effects were recognized by a value lower than the rate of the control. The developmental stages (fruiting body; sorophore and sorus) of *D. discoideum* NC4 as affected by each monoterpenoid were observed under an optical microscope (×50). The photos of fruiting bodies represent frequent results. All photographs were viewed in the same absolute scale and magnification to compare relative sizes between observations. For each treatment, there were four replicates. Data were analyzed by using ANOVA; LSD tests were performed for treatment effects at each concentration and the control.

RESULTS

Effect of Monoterpenes on D. discoideum *NC4 Growth.* The effect of monoterpenes on growth of *D. discoideum* NC4 are shown in Figure 2. All compounds at 1 μg/μl had a strong inhibitory effect on cell growth, resulting in a growth rate much lower than the control. With the exceptions of fenchone (at 0.001, 0.01, 0.1 μg/μl) and camphene (at 0.01 μg/μl), compounds showed a high inhibitory effect on growth. In contrast, fenchone and camphene stimulated growth (Figure 2). Even when there were no significant differences between some treatments and the control (for example, limonene at 0.01 μg/μl), the compounds had mild effects on *D. discoideum* NC4 cell growth; the shapes of cells were like the very short sorophores, as well as the smaller sized sorus compared to the control under the microscope (Figure 3). The rest of the compounds inhibited growth of *D. discoideum* NC4 cells including: geranyl acetate, (1*S*)-(−)-verbenone, (1*S*)-(−)-α-pinene, (+)-β-pinene, myrcene, (−)-menthone, (−)-bornyl acetate, (*S*)-(+)-carvone, (−)-camphene, and (*R*)-(+)-limonene. Microscope photos show these results (Figures 4 and 5).

Figure 3 shows the fruiting bodies of *D. discoideum* NC4 are affected by (1*R*)-(−)-fenchone and (−)-camphene. Very thick sorophores and a lager sori were formed in the presence of (1*R*)-(−)-fenchone at 1 and 0.1 μg/μl. Spores inside sori germinated as in the control. These microscopic observations matched growth rate results. There were spores in sori at all concentrations of (1*R*)-(−)-fenchone (a dark sorus indicates it is full of spores). However, we did not find spores in the sori at 1 and 0.1 μg/μl concentrations of camphene (a blight sorus indicates no spores). We confirmed the spores could be germinated. Figure 3 shows the effect of (−)-camphene on *D. discoideum* NC4 fruiting bodies. The size of the fruiting bodies at 1 and 0.1 μg/μl were much reduced, and the sori were empty.

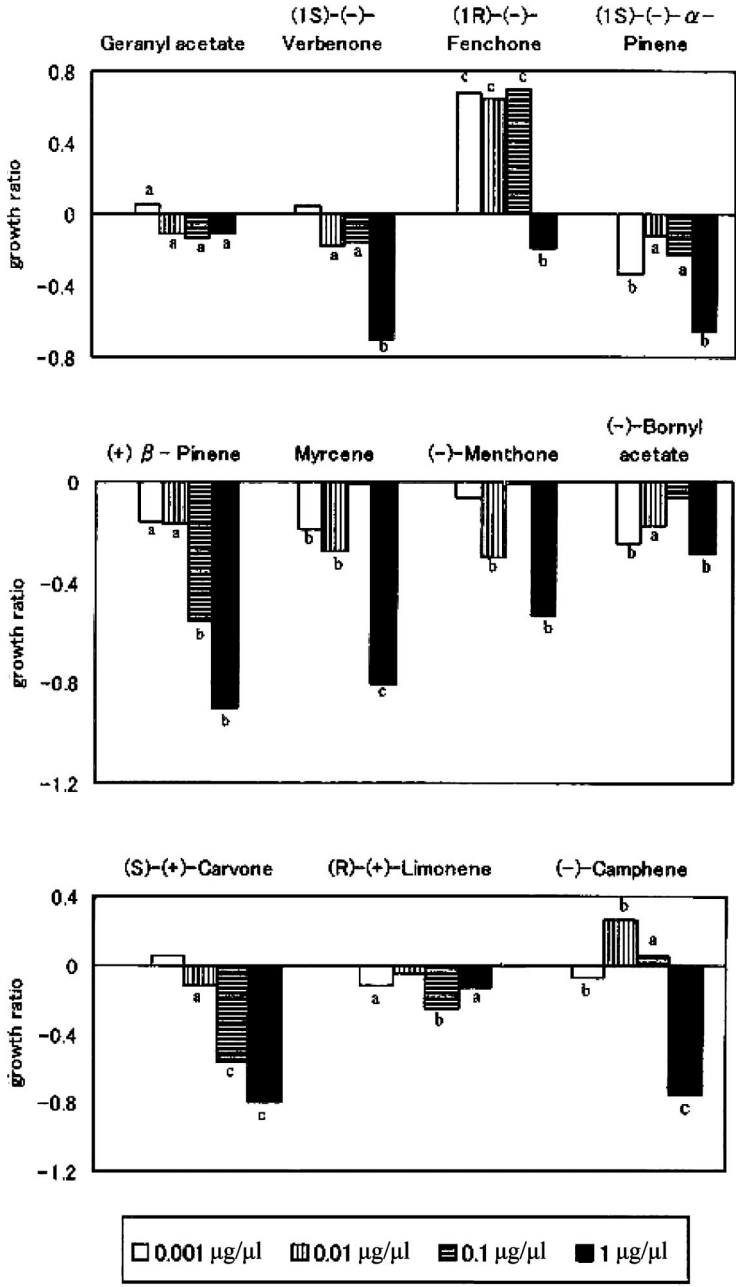

FIG. 2. Effect of monoterpenoids of *D. discoideum* NC4 growth rate. The control value is zero. Means designated with the same letter within a treatment are significantly different at $P < 0.05^{a}$, 0.01^{b}, and 0.001^{c} based on LSD test.

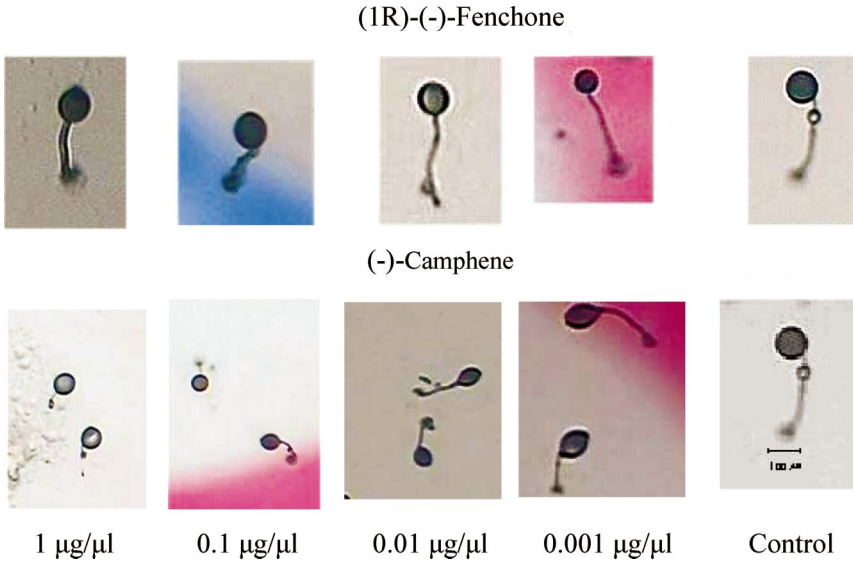

FIG. 3. Sizes and shapes of fruiting bodies of *D. discoideum* NC4 at four different concentrations of (1*R*)-(−)-fenchone and (−)-camphene. All photos are on the same scale as the control.

Although smaller than the control, the fruiting body sizes at 0.01 and 0.001 μg/μl were bigger than those at the other two concentrations. Sori were somewhat elongated and contained spores.

Figure 4 shows *D. discoideum* NC4 fruiting bodies are affected by geranyl acetate, (1*S*)-(−) verbenone, (1*S*)-(−)-α-pinene, β-pinene, and myrcene. Five compounds changed the shapes of fruiting bodies. They have short, slim sorophores, smaller sized sori of elliptical and/or abnormal shape, and no spores in them. β-pinene at 1 μg/μl and myrcene at 0.01 μg/μl especially, were different from the control. Notably, fruiting bodies at 1 and 0.1 μg/μl were not forced in the presence of (1*S*)-(−)-α-pinene.

Figure 5 shows *D. discoideum* NC4 is affected by (−)-menthone, (−)-bornyl acetate, (*s*)-(+)-carvone, and (*R*)-(+)-limonene. Fruiting bodies did not develop in the presence of (−)-bornyl acetate at 1 μg/μl (Figure 5). The sorophore was thick, unlike the control, with the same thickness as the sorus, which appears elongated. At 0.1 μg/μl of bornyl acetate, the fruiting bodies were a smaller than controls and with shorter sorophores. Specifically, the shape of sori was not circular and contained no spores. (*S*)-(+)-Carvone also affected the fruiting bodies. At all concentrations, (*S*)-(+)-carvone caused similarly shaped fruiting bodies, which were all smaller than the control. The effect of carvone was seen even at the lowest concentration examined.

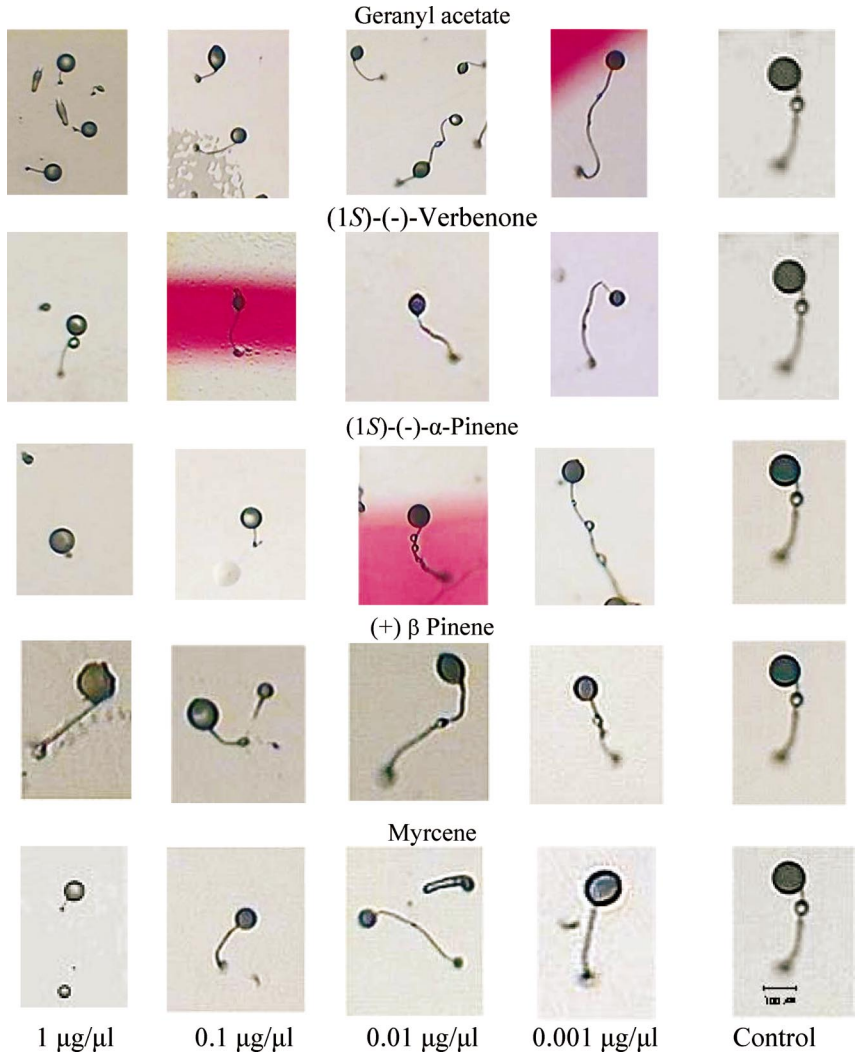

FIG. 4. Sizes and shapes of fruiting bodies of *D. discoideum* NC4 at four different concentrations of geranyl acetate, (1*S*)-(−)-verbenone, (1*S*)-(−)-α-pinene, (+)-β-pinene, and myrcene. All photos are on the same scale as the control.

DISCUSSION

With the exceptions of fenchone and camphene, all examined compounds showed inhibitory effects on the growth of *D. discoideum* NC4. Figures 4 and 5

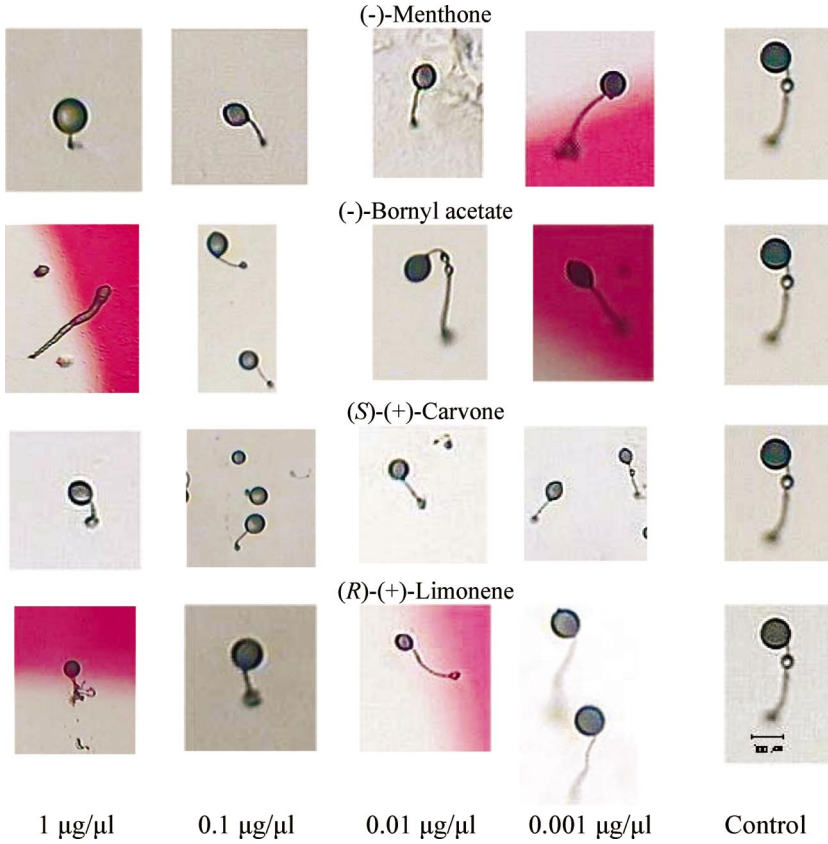

FIG. 5. Various sizes and shapes of fruiting bodies of *D. discoideum* NC4 at four concentrations of menthone, bornyl acetate, (*S*)-(+)-carvone, and limonene. All photos are on the same scale as the control.

suggest that these compounds influence the shape of fruiting bodies; shorter and thinner sorophores and smaller elliptical sori are formed in many cases without spores. In contrast, fenchone and camphene (at three and two concentrations, respectively) stimulated the growth of *D. discoideum* NC4. In addition, we found that enhancement of the growth of *D. discoideum* NC4 was accompanied by the generation of thick sorophores and larger sori (Figure 3). Vokou et al. (2002) reported that fenchone, the major compound from *Lanvandula stoechas*, inhibited *Bacillus subtilis*, but did not inhibit *Escherichia coli* or any of the soil microorganisms used in their study. They suggested that *E. coli* and soil microorganisms used fenchone as carbon and energy sources. Cleveland and Yavitt (1998) also reported that soil microorganisms in temperate forest soil consume isoprene even

when present in small amounts. Our experiments were not designed to examine whether these compounds can be used as an energy source. Nevertheless, on the basis of our results, we could argue that fenchone and camphene are potential carbon sources for *D. discoideum* NC4, as suggested by Vokou et al. (2002).

The lack of spores in sori suggests that even the next generation is likely to be affected by the compounds. Many researchers have reported that monoterpenes produced by plants affect the growth of microorganisms. Oosterhaven et al. (1996) reported that (+) carvone has a strong antifungal activity. The volatile oils of the species tested (*Thymus vulgaris*, *Origanum vulgare*, *Origanum dictamus*, and *Artemisia princeps*) inhibited fungal spore gemination and mycelial growth (Daferera et al., 2000; Yun and Choi, 2002). CSMs produce substances (so-called slime) on the outside of their sori when they aggregate. These substances protect spore dispersion until they are large enough to germinate. If the normal sorus is touched with a needle, the sorus is destroyed and the spore germinates. However, when we touched monoterpenoid impacted sori, they remained intact. These results suggest that monoterpenes primarily affect internal events in *D. discoideum* NC4. Most of the inhibitory effects were represented by smaller and strangely shaped sori without spores, even at the lowest concentration of each compound tested.

Little is known about monoterpene distribution in soils and their effects on soil microorganisms, although a number of studies have proposed them (Kainulainen and Holopainene, 2002). Nevertheless, there is substantial evidence that individual monoterpenes may have entirely different activating effects on different target microorganisms. Our results show that *D. discoideum* NC4 is not inhibited by fenchone and camphene, and perhaps utilizes them as carbon and/or energy sources. All other monoterpenoids we tested inhibited the growth of *D. discoideum* NC4. The population of CSM is poor in pine forest floors (Feest and Madelin, 1988). One of the reasons might be the inhibitory effect of many monoterpenes on their growth. This is the first study examining the effect of monoterpenoids on CSMs. Further research is needed to examine whether this activity plays a role in the composition and abundance of soil microorganism and further on the decomposition process in pine forest floors.

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VARIATION OF BROMINATED INDOLES AND TERPENOIDS WITHIN SINGLE AND DIFFERENT COLONIES OF THE MARINE BRYOZOAN *Flustra foliacea*

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Abstract—The variation of the brominated indole and diterpenoid content within single and different colonies of the bryozoan *Flustra foliacea* was investigated. The secondary metabolite profile and concentrations of individual components of *F. foliacea* samples were established using GC–MS. Samples from 17 different collecting sites were analyzed. The alkaloid and diterpene composition of *F. foliacea* varied greatly depending upon the site of collection. Investigation of *F. foliacea* samples from a single site (Helgoland, North Sea) over a period of time showed that the alkaloid and diterpenoid profile remained constant, however concentrations of individual components varied significantly. The alkaloid and diterpenoid composition of different segments of a single colony was found to be constant. Only small differences could be detected in the essential oil composition of different colonies and segments of single colonies of *F. foliacea*. Two of the *F. foliacea* alkaloids were found in the gastropods *Hydrobia ulva* and *Gibbula cinerea*, and one alkaloid in the common starfish *Asteria rubens*, all collected from the surface of the bryozoan.

Key Words—*Flustra foliacea*, bryozoan, alkaloid, terpene, distribution, GC–MS.

INTRODUCTION

Flustra foliacea is a common bryozoan occurring in the North Sea, Nova Scotia (Canada), in the White Sea (Russia), and various other locations. To date, 17 alkaloids, including 16 indoles and one quinoline, have been isolated from the bryozoan collected in the North Sea (Christophersen, 1991; Holst et al., 1994a,b; Peters et al., 2002), and five structurally related, but different, indole alkaloids

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from animals of Canadian waters (Wright, 1984; Laycock et al., 1986). In addition, alkaloids several monoterpenes, i.e., *cis*- and *trans*-citral, citronellol, nerol, geraniol, rosefuran, 3-(4-methyl-3-pentenyl)furan, and citronellal were found in *F. foliacea* (Christophersen and Carlé, 1978; Holst et al., 1994b).

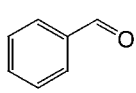
The isolation of alkaloids with differing structures from *F. foliacea* colonies obtained from distant collecting sites points to some variation of alkaloid patterns among *F. foliacea* populations. Earlier investigations indicated *F. foliacea* metabolites may be of ecological significance. Al-Ogily and Knight-Jones (1977) observed that older parts of the fronds of *F. foliacea* inhibited the growth of *Staphylococcus aureus* to a greater extent than parts of the growing edge, and also showed a much stronger and characteristic smell of lemons, suggesting some variation of the secondary metabolite content within a single *F. foliacea* colony. Also, dihydroflustramine C (**12**) and flustramine D (**18**) have been found to be capable of antagonizing acylhomoserine lactone(AHL)-dependent quorum sensing systems (Peters et al., 2003).

The aim of the current study was to determine the variation of the secondary metabolite content (see Figure 1) within single and different colonies of *F. foliacea* as a basis for further detailed studies into the chemical ecology of this marine animal.

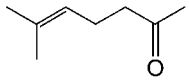
METHODS AND MATERIALS

GC–MS Analysis. GC–MS results were obtained on a Perkin–Elmer AutoSystem XL with a Perkin–Elmer TurboMass detector and autosampler at 70 eV, 1 scan/sec at 180°C detector temperature, m/z 50–650 TIC-mode, injection volume 1 μl, split ratio 1/5. GC separation was carried out by using a Perkin–Elmer PE-1 column (30 m $\times$ 0.32 mm id), and He (2 ml/min) as carrier gas. Perkin–Elmer software TurboMass version 4.1.1. was applied. For GC separation of alkaloids and diterpenes, a temperature gradient was used (90–160°C at 6°C/min, then 160–300°C at 10°C/min). For GC separation of essential oil constituents, the temperature gradient was 50–230°C at 4°C/min, then 230–300°C at 10°C/min. Compounds were identified by direct comparison of their retention times and mass spectral data with those of authentic samples (Peters et al., 2002, 2003) or literature data (Carlé and Christophersen, 1979, 1980, 1981; Melkani et al., 1994) (Table 1). Injection of the internal standard (IS) 1-bromohexadecane was used to validate retention times.

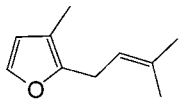
Relative concentrations (in percent) were determined by examining the contribution of each peak to the total area of all relevant compounds. Peaks contributing less than 0.1% to the overall profile were excluded from all analyses. Absolute concentrations were quantified via a one-point calibration. For every pure compound (**11–21**, see Figure 1), the detector response $A_{\text{com}}/A_{\text{IS}}$ (A = peak area) was



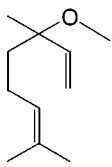
Benzaldehyde (**1**)



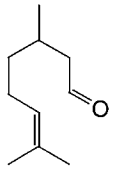
6-Methyl-5-hepten-2-one (**2**)



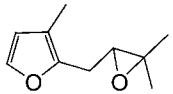
Rosefuran (**3**)



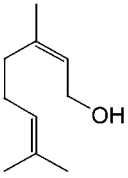
Linalool (**4**)



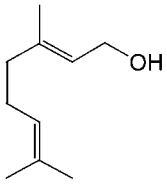
Citronellal (**5**)



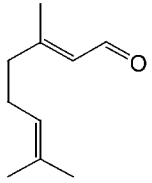
Rosefuran epoxide (**6**)



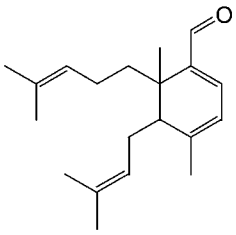
Nerol (**7**)



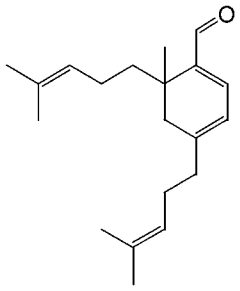
Geraniol (**8**)



Geranial (**9**)

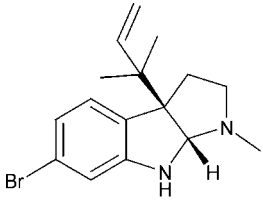


1,4,5-Trimethyl-6-(3-methyl-2-butenyl)-5-(4-methyl-3-pentanyl)-1,3-cyclohexadiene (**10**)

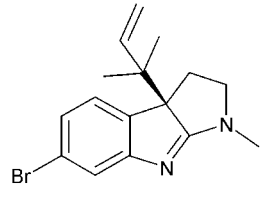


4,6-Bis(4-methylpent-3-en-1-yl)-6-methylcyclohexa-1,3-diene-carbaldehyde (**11**)

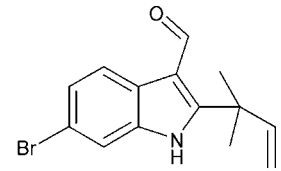
FIG. 1. Structures (relative stereochemistry) of natural products in *Flustra foliacea*.



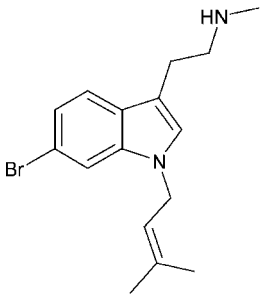
Dihydroflustramine C (**12**)



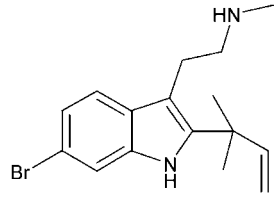
Flustramine C (**13**)



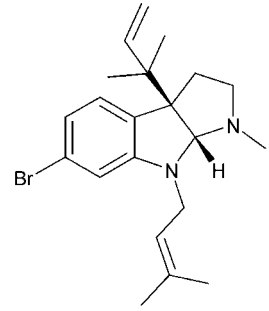
6-Bromo-2(1,1-dimethyl-2-propenyl)-1*H*-indole-3-carbaldehyde (**14**)



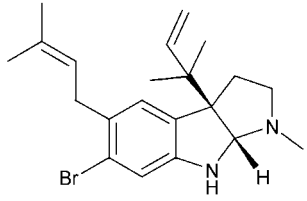
Deformylflustrabromine B (**15**)



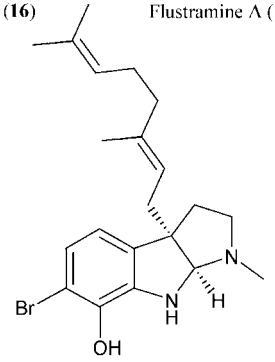
Deformylflustrabromine (**16**)



Flustramine A (**17**)

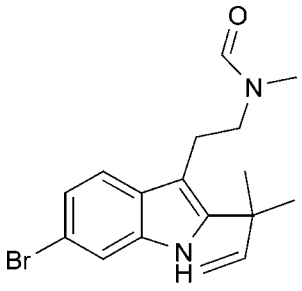


Flustramine D (**18**)

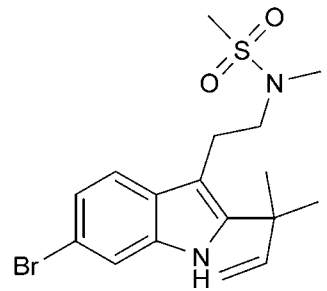


(3a*R**,8a*S**)-6-Bromo-3a-[(2*E*)-3,7-dimethyl-2,6-octadienyl]-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-*b*]indol-7-ol (**19**)

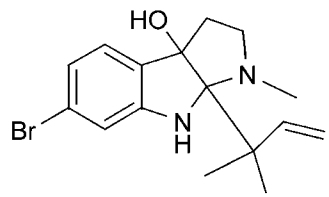
FIG. 1. Continued



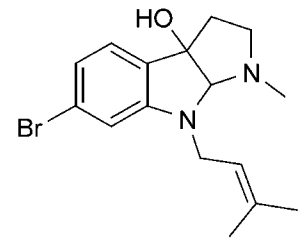
Flustrabromine (**20**)



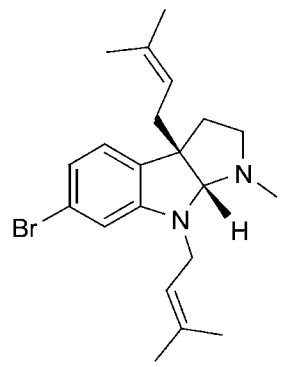
N-(2-[6-Bromo-2-(1,1-dimethyl-2-propenyl)-1*H*-indol-3-yl]ethyl)-N-methylmethanesulfonamide (**21**)



Flustraminol A (**22**)



Flustraminol B (**23**)



Flustramine B (**24**)

FIG. 1. Continued

TABLE 1. GC–MS ANALYSIS OF *Flustra foliacea* METABOLITES

RT[a] (min)	Coinjection of authentic sample	Confirmed with literature data	Compound
3.5[b]	Yes		Benzaldehyde (**1**)
5.4[b]	Yes		6-Methyl-5-hepten-2-one (**2**)
8.5[b]	Yes		Rosefuran (**3**)
8.6[b]	Yes		Linalool (**4**)
10.0[b]	Yes		Citronellal (**5**)
10.7[b]		Yes[d]	Rosefuran epoxide (**6**)
12.6[b]	Yes		Nerol (**7**)
13.4[b]	Yes		Geraniol (**8**)
13.5[b]	Yes		Geranial (**9**)
17.0[c]	Yes		1,4,5-Trimethyl-6-(3-methyl-2-butenyl)-5-(4-methyl-3-pentanyl)-1,3-cyclohexadiene (**10**)
17.6[c]	Yes		4,6-Bis(4-methylpent-3-en-1-yl)-6-methylcyclohexa-1,3-diene-carbaldehyde (**11**)
17.9[c]		Unidentified	*m*/*z* = 251/253
18.0[c]	Yes		Dihydroflustramine C (**12**)
18.3[c]		Unidentified	*m*/*z* = 306/308
18.3[c]		Unidentified	*m*/*z* = 320/322
18.5[c]		Yes[e]	Flustraminol B (**23**)
18.7[c]	Yes		Flustramine C (**13**)
19.3[c]		Yes[e]	Flustraminol A (**22**)
19.8[c]	Yes		6-Bromo-2(1,1-dimethyl-2-propenyl)-1H-indole-3-carbaldehyde (**14**)
19.9[c]	Yes		Deformylflustrabromine B (**15**)
20.0[c]	Yes		Deformylflustrabromine (**16**)
20.6[c]	Yes		Flustramine A (**17**)
20.7[c]		Yes[e]	Flustramine B (**24**)
21.1[c]		Unidentified	*m*/*z* = 331/333
21.3[c]	Yes		Flustramine D (**18**)
21.5[c]		Unidentified	M/z = 404/406
22.1[c]		Unidentified	M/z = 404/406
22.4[c]	Yes		(3a*R**,8a*S**)-6-Bromo-3a-[(2*E*)-3,7-dimethyl-2,6-octadienyl]-1,2,3,3*a*,8,8*a*-hexahydropyrrolo[2,3-b]indol-7-ol (**19**)
23.1[c]	Yes		Flustrabromine (**20**)
24.3[c]	Yes		*N*-(2-[6-Bromo-2-(1,1-dimethyl-2-propenyl)-1H-indol-3-yl]ethyl)-*N*-methylmethanesulfonamide (**21**)

[a] RT = Retention time.
[b] GC–MS of essential oil constituents: 50–230°C at 4°C/min; 230–300°C at 10°C/min.
[c] GC–MS of alkaloids and diterpenes: 90–160°C at 6°C/min; 160–300°C at 10°C/min.
[d] Melkani et al., 1994.
[e] Carlé and Christophersen, 1979, 1980, 1981.

TABLE 2. COLLECTION SITE OF *Flustra foliacea* SAMPLES

Voucher no.	Date of collection (month.year)	Location	Depth
Wal1	10.2001	Harwich/Wales	Beach
Wal2	10.2001	Menai Strait/Wales	Beach
Eng1	1990–1996	Northumberland	—
Rus1	07–08.2002	66.20N, 33.40O	15–20 m
Can1	06.1973	NB-Petersongrab/Lorneville	—
NS1	08.2001	58.25N, 1.5W/North Sea	15–20 m
NS2	08.2001	56.75N, 4.5E/North Sea	15–20 m
NS3	09.2001	59.25N, 1.5W/North Sea	15–20 m
NS4	09.2001	59.25N, 1.5W/North Sea	15–20 m
NS5	06.2002	53.55N, 9.45E/North Sea	15 m
ST1	05.1998	Steingrund/Helgoland	15 m
ST2	08.1998	Steingrund/Helgoland	15 m
ST3	05.2001	Steingrund/Helgoland	15 m
ST4	08.2001	Steingrund/Helgoland	15 m
Hel1	09.1998	Southwest side/Helgoland	12 m
Hel2	09.1998	Northwest side/Helgoland	12 m
Hel3	07.2000	West side/Helgoland	11 m

measured (three replicates) at a concentration of 1.0 mg/ml. The mean value of the detector response was used to calculate the concentration of compounds **11–21** on a wet weight basis.

Bryozoan Collections. Seventeen bryozoan samples were collected from various sites in the North Sea, off Russia, and off Atlantic Canada (Table 2) to investigate site-specific variation in the alkaloid and diterpenoid content. Epibionts were removed from all colonies from Helgoland (including Steingrund), and the samples were transported at 0°C and stored at −20°C. All other colonies were directly stored in ethanol or isopropanol.

Variation in alkaloid and diterpenoid content over time was investigated with five samples (HT04 to HT09) from the West side of Helgoland (depth 11 m) over a period of 5 months (4–2001 to 9–2001).

Extraction Methods. Three to five colonies (1.0 g wet weight, Table 2) of *F. foliacea* were extracted ×3 with 20 ml freshly distilled dichloromethane. After evaporation of the solvent, the extract was dissolved in 1.0-ml dichloromethane with an internal standard (1-bromohexadecane) concentration of 0.01 mg/ml. Because of small amounts of sample, in the case of Rus1 and Can1, only 0.2 g wet weight and 0.5 g wet weight, respectively, of *F. foliacea* were used for these extractions. To detect minor metabolites, a second extraction with an increased amount of sample was performed (e.g., Can1: 1.5 g, Rus1: 0.6 g, Eng1: 5.0 g), and the extract was dissolved in 20 μl dichloromethane.

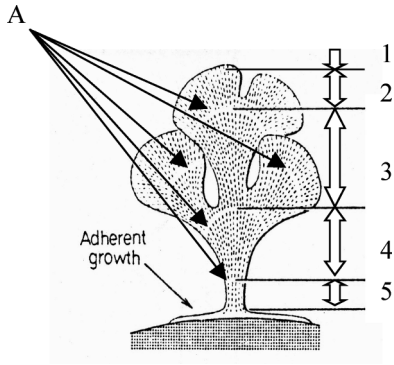


FIG. 2. Diagram of *Flustra foliacea* frond showing growth check lines (A), and delineating segments 1 to 5 (adapted from Stebbing, 1971).

Metabolite Distribution Within a Single Frond of a Colony of F. foliacea. Two individual fronds of a colony (ST3) were separately cut into five segments according to the growth check lines (Figure 2). Wet weight 0.2 g of every segment was extracted $\times 3$ with 20 ml freshly distilled dichloromethane. After removal of the solvent, the extract was dissolved in 1.0-ml dichloromethane containing an internal standard concentration of 0.01 mg/ml. Two samples from Helgoland, Hel3, and ST3 were taken to investigate the essential oil distribution within a single frond. The frond was cut into five segments according to the growth check lines (Figure 2). From every segment, 0.7 g wet weight material were extracted using water steam distillation (Karlsruher instrument). For this purpose, 100 ml water were added to the sample. The volatiles were absorbed in 0.3 ml of hexane functioning as a receiver, over 4 hr with a distillation speed between 2 and 3 ml/min. The resulting extract was diluted with hexane up to a volume of 1.0 ml, including 0.01 mg of the internal standard 1-bromohexadecane.

Uptake of Alkaloids by Grazing Predators. To investigate the uptake of alkaloids by predators grazing on the surface of *F. foliacea*, two snails (*Hydrobia ulva*, 0.5 g wet weight; *Gibbula cinerea*, 0.6 g wet weight), a nudibranch (*Cuthona amoena*, 0.1 g wet weight), and a starfish (*Asteria rubens*, 5.0 g wet weight) were collected from the surface of *F. foliacea*. The samples were extracted $\times 3$ with 20 ml freshly distilled dichloromethane. After removal of the solvent, the extract was dissolved in 10-μl dichloromethane and injected directly onto the GC-column (2 μl).

Release of Alkaloids from F. foliacea into the Surrounding Water. To investigate the release of alkaloids from *F. foliacea* into the surrounding water, 200 g of XAD16 (Fluka) were used as an adsorbent. Water at the rate of 400 ml/hr, of a 20-l aquarium containing an aquaculture of *F. foliacea*, were pumped through

the adsorbent for 1 week. XAD16 was extracted $\times 3$ with 200 ml freshly distilled methanol. After removal of the solvent, the extract was dissolved in 1.0-ml methanol and injected directly onto the GC-column (1 μl).

Statistical Analysis. The software SAS (Statistical analysis system) was used. A one-way ANOVA and a GLM analysis was performed with the data shown in Table 4.

RESULTS

Alkaloid and Diterpene Content of F. foliacea *Populations Dependent on the Site and Time of Collection*. *F. foliacea* colonies from the different collection sites produced 18 structurally different bromoalkaloids. Of these, six metabolites are as yet unidentified alkaloids that characterized only through their specific MS isotope pattern for bromine containing compounds. For example, GC–MS chromatograms show the number, identity, and relative amounts of individual diterpenes and alkaloids present in different *F. foliacea* populations (Figure 3a and b, Table 3). The number of bromoalkaloids (Figure 1) detected in each sample varies greatly from 11 in NS4 and Hel3 to none in the sample from Canada (Can1, dependent on the detection limit, see below). NS1 from the North Sea contained only flustramine A (**17**), and the sample Eng1 yielded merely one unidentified bromoalkaloid. All samples, except those from Helgoland (Hel1, Hel2, Hel3, ST1, ST2, ST3, and ST4), produced one major metabolite accounting for more than 50% of the alkaloid and diterpenoid content. Dihydroflustramine C (**12**) is the major alkaloid of the Harwich (Wal1) and North Wales (Wal2) samples, formerly also described as being present in a Canadian (Bay of Fundy, New Brunswick, Nova Scotia) sample (Laycock et al., 1986). The investigation of samples from seven closely related collection sites around Helgoland showed the alkaloid and diterpenoid content to be similar. In these samples, deformylflustrabromine (**16**) and flustramine A (**17**) are the major metabolites. Unlike all other samples having alkaloids as predominant metabolites, NS5 from the North Sea contained diterpene **11** as its major metabolite.

Increasing the concentration of the extract to detect minor metabolites resulted in the isolation of additional bromine-containing compounds: In the case of Can1, three unidentified bromosubstituted structures were isolated; in the case of Rus1, flustramine A (**17**) and dihydroflustramine C (**12**) were identified and three unidentified bromosubstituted compounds were isolated; and in the case of Eng1, dihydroflustramine C (**12**) was identified and 10 unidentified bromine-containing metabolites were isolated.

Time of collection was also an important factor when considering alkaloid and diterpenoid patterns. Samples from the West side of Helgoland (HT04 to HT09) were obtained from April to September 2001. In the winter months, no samples

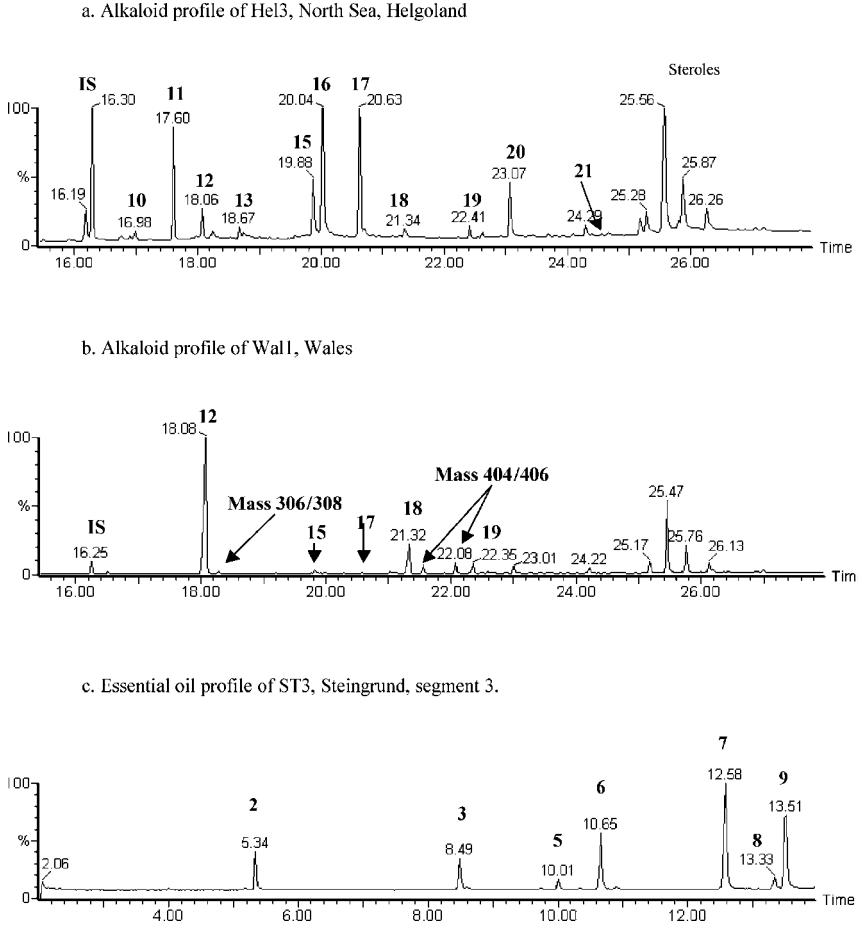


FIG. 3. GC chromatograms of *Flustra foliacea* samples.

could be collected due to unfavorable weather conditions. Nine bromoalkaloids were identified in these samples by comparison of GC retention times and MS data with those of authentic compounds. The detector response of every compound was established in order to calculate the absolute concentration of compounds (**11–13, 15–21**, structures see Figure 1) on a wet weight basis. Each of the samples contained significant amounts of the diterpenoid dicitral A (**11**). The major alkaloid in all samples was deformylflustrabromine (**16**). The qualitative secondary metabolite pattern did not vary with time (GLM procedure), however, there were statistically (ANOVA) significant changes regarding the quantity of individual alkaloids and dicitral (**11**). For compounds (**11**, **15**, **16**, and **21**), concentrations increased from

TABLE 3. GEOGRAPHICAL VARIATION OF ALKALOID AND DITERPENOID CONTENT OF *Flustra foliacea* COLONIES[a,b]

	Wal1[c]	Wal2[c]	NS1[c]	NS2[c]	NS3[c]	NS4[c]	NS5[c]	Hel3[d,e]	Eng1[d]	Rus1[c]
Dicitral B (**10**)	—[f]	—	—	—	—	—	4.2	1.1	—	—
Dicitral A (**11**)	—	—	—	—	6.3	7.1	52.1	12.4	—	—
m/*z* = 251/253	—	—	—	—	—	—	—	—	—	50
Dihydroflustramine C (**12**)	78.6	73.8	—	—	—	0.9	—	3.7	—	—
m/*z* = 306/308	0.8	—	—	—	—	—	—	—	—	—
m/*z* = 320/322	—	—	—	15.9	8.8	10.6	—	—	—	—
Flustraminol B[g] (**23**)	—	—	—	2.6	1.4	1.8	—	—	—	—
Flustramine C (**13**)	—	—	—	—	—	—	—	1.6	—	—
Flustraminol A[g] (**22**)	—	—	—	6.8	0.8	1.1	—	—	—	—
Deformylflustrabromine B (**15**)	1.5	—	—	—	0.6	0.8	—	7.8	—	—
Deformylflustrabromine (**16**)	—	—	—	3.4	1.3	1.0	—	36.2	—	—
Flustramine A (**17**)	0.4	19.8	100	4.7	8.0	11.5	30.2	26.4	—	—
Flustramine B[g] (**24**)	—	—	—	66.6	71.7	64.7	—	—	—	50
Flustramine D (**18**)	11.3	—	—	—	—	—	5.8	1.8	—	—
m/*z* = 331/333	—	—	—	—	—	—	—	—	100	—
m/*z* = 404/406	2.2	2.4	—	—	0.4	0.2	—	—	—	—
m/*z* = 404/406	2.7	4.1	—	—	0.3	0.1	—	—	—	—
Compound **19**	2.5	—	—	—	0.4	0.4	7.7	1.5	—	—
Flustrabromine (**20**)	—	—	—	—	—	—	—	7.1	—	—
Compound **21**	—	—	—	—	—	—	—	0.6	—	—

[a] Relative amounts (%) $= A_{\text{com}} / \sum A_{\text{com}} \cdot 100$. The mean value of the standard deviation was 14%.
[b] No alkaloids and diterpenes were detected during this study in the sample Can1, since their concentration was below the detection limit (see Results).
[c] $N = 3$.
[d] $N = 5$.
[e] Further samples (Hel1, Hel2, ST1, ST2, ST3, and ST4) from other collection sites around Helgoland are closely related.
[f] not detected.
[g] Mass spectroscopic data in good agreement with literature data (Carlé and Christophersen, 1979, 1980, 1981).

April to June. This was followed by a decrease in August and an increase in September. The content of compounds **17**, **19**, and **20** decreased from April to May. The concentration of these compounds then increased in June and decreased until September. The amount of compounds **12** and **18** increased from April to June and then decreased until September. The concentration of compound **13** increased from April to September. A pronounced increase in concentration was noticed for the diterpene **11** from spring to autumn (170 μg/g wet weight to 456 μg/g wet weight) (Table 4).

Alkaloid and Diterpene Distribution Within a Single Frond of a Colony of F. foliacea. The relative concentration and composition of the alkaloid and diterpenoid content of a single colony of *F. foliacea* (ST3) was investigated. For this

TABLE 4. ALKALOID AND DITERPENOID CONTENT OF *Flustra foliacea* SAMPLES FROM HELGOLAND, DETERMINED AT DIFFERENT TIMES OF THE YEAR

	HT04[a]	HT05[b]	HT06[b]	HT08[b]	HT09[b]
Dicitral A (**11**)	170[c]	209	409	328	456
Dihydroflustramine C (**12**)	4	11	12	6	5
Flustramine C (**13**)	2	3	3	4	4
Deformylflustrabromine B (**15**)	38	37	56	24	39
Deformylflustrabromine (**16**)	248	232	358	165	364
Flustramine A (**17**)	29	13	44	35	34
Flustramine D (**18**)	5	10	11	5	8
Compound **19**	16	12	44	19	15
Flustrabromine (**20**)	44	34	65	49	42
Compound **21**	7	25	53	10	24
Total amount of alkaloids	393	377	646	317	535

[a] $N = 3$.
[b] $N = 5$.
[c] Concentration (c) in μg/g fresh weight, $c = \frac{(A_x/A_{IS})(A_{IS_{calibration}}/A_{x\ calibration})}{\text{wet weight (g/ml)}} \cdot m_{IS}$, A_x = area of compound (**11–13**, **15–21**); A_{IS} = area of internal standard; m_{IS} = weight internal standard = 100 μg/ml; the mean value of the standard deviation was 9%.

purpose, the fronds of the colony were dissected according to the growth lines as shown in Figure 2. GC–MS analysis of the dichloromethane extract showed that the alkaloid and diterpenoid pattern of the different segments is, close to identical, and quantitatively similar, except for compound **13** (Table 5).

Essential Oil Distribution Within Different Segments of a Colony and Within Colonies of F. foliacea. The low amount of essential oil components in *F. foliacea*

TABLE 5. ALKALOID AND DITERPENOID COMPOSITION OF DIFFERENT SEGMENTS OF A FROND OF *F. foliacea* FROM HELGOLAND (ST3)[a]

	Segment 1	Segment 2	Segment 3	Segment 4	Segment 5
Dicitral (**11**)	7	2	2	2	1
Dihydroflustramine C (**12**)	6	11	7	7	8
Flustramine C (**13**)	<0.5	<0.5	3	2	10
Compound **14**	2	1	4	1	1
Deformylflustrabromine B (**15**)	5	8	6	7	4
Deformylflustrabromine (**16**)	54	65	52	57	57
Flustramine A (**17**)	13	8	10	7	5
Flustramine D (**18**)	<0.5	<0.5	<0.5	<0.5	<0.5
Compound **19**	<0.5	<0.5	<0.5	<0.5	<0.5
Flustrabromine (**20**)	9	2	7	7	4
Compound **21**	2	1	2	3	2

[a] Relative amounts (%) = $A_{com}/\sum A_{com}$ (**11–21**) $\cdot$ 100.

TABLE 6. ESSENTIAL OIL COMPOSITION OF *F. foliacea* COLONIES FROM THE WEST SIDE (HEL3) AND FROM THE STEINGRUND (ST3) OF HELGOLAND[a]

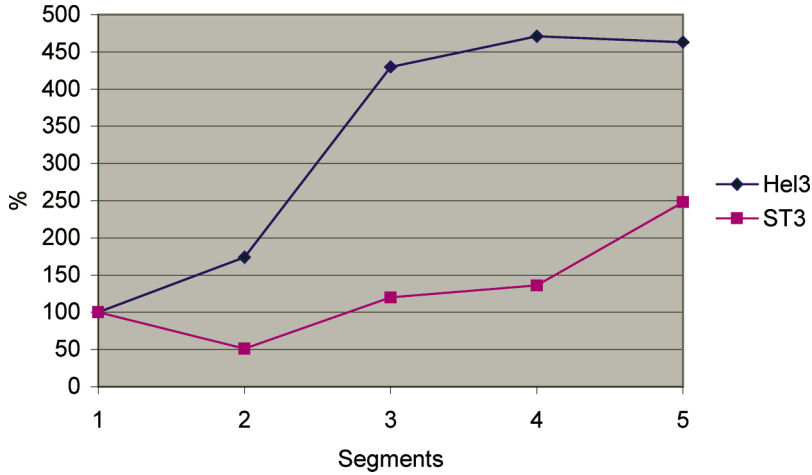
	Segment 1		Segment 2		Segment 3		Segment 4		Segment 5	
	ST3	Hel3	ST3	Hel3	ST3	Hel3	ST3	Hel3	ST3	Hel3
Benzaldehyde (**1**)	<0.5	<0.5	<0.5	<0.5	<0.5	4.7	<0.5	7.7	<0.5	7.5
6-Methyl-5-hepten-2-one (**2**)	6.3	14.9	11.0	20.0	9.8	13.3	6.6	20.8	8.5	20.7
Rosefuran (**3**)	4.9	5.4	8.1	4.4	7.7	2.6	6.3	4.8	3.7	6.7
Linalool (**4**)	<0.5	<0.5	<0.5	0.8	<0.5	<0.5	<0.5	2.3	1.5	<0.5
Citronellal (**5**)	5.2	14.5	5.9	2.4	3.1	5.9	2.5	2.4	1.5	1.0
Rosefuran epoxide (**6**)	4.2	3.0	13.1	6.4	15.9	9.2	16.6	17.0	14.0	10.8
Nerol (**7**)	46.3	38.6	34.2	34.8	34.8	53.1	37.0	21.9	29.0	22.5
Geraniol (**8**)	<0.5	1.2	3.2	4.8	3.7	0.7	4.1	1.3	16.6	2.2
Geranial (**9**)	33.1	22.4	24.5	26.5	25.1	10.5	26.9	21.9	28.5	29.4

[a] Relative amounts (%) $= A_{\text{com}} / \sum A_{\text{com}}$ (**1–9**) $\cdot$ 100.

did not allow their direct detection after extraction with lipophilic solvent, but required a special enrichment. Steam distillation was used to concentrate these metabolites. The essential oil concentration and composition of different segments of *F. foliacea* (Hel3 and ST3) from two different locations was analyzed by GC–MS (Figure 3c). The composition of the essential oil of the different segments and the different colonies was similar (Table 6). The major metabolites were nerol (**7**) and geranial (**9**) in both colonies. In both colonies, the concentration of the essential oil increased from the colony tip, i.e., segments 1 and 2 to the base segment 5, following the age of the tissue (Figure 4). The essential oil concentration at the base of the two colonies was two to four times higher than that in the tips (data not shown). Minor changes among the colonies were however detectable, e.g., benzaldehyde (**1**) was only present in Hel3, and 6-methyl-5-hepten-2-one (**2**) was present in significantly higher concentration in Hel3 as compared to ST3. The relative essential oil concentration in both colonies can be judged by comparing the sum of all relevant chromatographic peaks (**1–9**). In Hel3 (26070 area units), the essential oil concentration is approximately 27% less than in ST3 (35674 area units). The small data set for the essential oil measurement ($N = 2$) excluded a statistical treatment of the data.

Uptake of Alkaloids by Grazing Predators. Grazers on the surface of *F. foliacea* seem to assimilate alkaloids. The alkaloids dihydroflustramine C (**12**) and flustramine A (**17**) were shown to be present in the snail *Hydrobia ulva* and *Gibbula cinerea*. The starfish *Asteria rubens* contained dihydroflustramine C (**12**). Extraction of the nudibranch *Cuthona amoena* yielded no *F. foliacea* metabolites.

Release of Alkaloids from F. foliacea *into the Surrounding Water*. To investigate the release of secondary metabolites from *F. foliacea* colonies into the



[a] Segments, see Figure 2

$$\frac{\frac{\sum A_{com1-9x}}{A_{IS}x}}{\frac{\sum A_{com1-9Seg.1}}{A_{IS}Seg.1}} \cdot 1000 = \%$$

A_{com} = area of compound (**1-9**) in a certain segment x
A_{IS} = area of internal standard
x = refers to number of Segment, i.e. 1, 2, 3, 4, 5 (see Figure 2)
100% = Σ Area of compounds **1-9** of segment 1.

FIG. 4. Relative essential oil concentration of different segments of single fronds of two *Flustra foliacea* colonies (Hel3, ST3).

surrounding water, the water of colonies maintained in aquaculture (Terramare, Wilhelmshaven) was filtered through an adsorbent (XAD16). Solid phase extraction of XAD16 identified the alkaloids flustramine C (**13**) and flustramine A (**17**) as being present in the surrounding water.

DISCUSSION

Former preparative HPLC investigations of the bryozoan *F. foliacea* from North Sea locations yielded 12 brominated indol alkaloids and two diterpenes, four of which were new structures (Peters et al., 2002, 2003; Peters, L., Wright, A. D., Kehraus, S., Gündisch, D., Tilotta, M. C., and König, G. M., submitted. Prenylated indole alkaloids from *Flustra foliacea* with subtype specific binding on nAChRs. *J. Nat. Prod.* submitted for publication). HPLC-DAD and HPLC-MS analysis of the lipophilic extracts allowed the detection of these compounds but provided insufficient resolution for the purpose of quantification. Thus, GC–MS methods, known for their high resolution power, were established here to quantify brominated alkaloids, diterpenes, and essential oil constituents (Table 3, Figure 3).

F. foliacea colonies at a certain location possess a distinct and fairly constant alkaloid and essential oil profile. In contrast, colonies from distant locations differ significantly from each other. Seasonal changes do occur, but only concerning the concentration of individual compounds. There are also differences in the essential oil concentration within a single frond of a colony, but the overall secondary metabolite profile, is constant.

These results are comparable to those originating from an investigation of the Tasmanian bryozoan *Amathia wilsoni* (Blackmann and Fu, 1989). *A. wilsoni* produces a series of secondary metabolites called amathamide A-F (Blackmann and Mathews, 1985; Blackmann and Fu, 1989). Blackmann and Green (1987) demonstrated that *A. wilsoni* collected from geographical different locations gave varying alkaloid patterns, in contrast to different colonies from the same location (Blackmann and Green, 1987; Walls et al., 1991). Another bryozoan with varying secondary metabolite content depending on the site of collection is *Bugula neritina*, the source of the bryostatins, known for their potent antitumoral activity. Davidson and Haygood (1999) defined two chemotypes on the basis of the occurrence of certain types of bryostatins. Thus, chemotype O contains bryostatins with an octa-2,4-dienoate substituent at C-20 of the bryopyran ring, but chemotype M lacks this substituent. These chemotypes correlate with genetic differences in the mitochondrial cytochrome *c* oxidase subunit 1 (CO I) gene of different *B. neritina* populations. Each chemotype was also found to harbor a different strain of the symbiont *Endobugula sertula. E. sertula* strain D (deep) is present in chemotype O, whereas strain S (shallow) is found in chemotype M (Davidson and Haygood, 1999).

Taking literature reports and our results into account, there are various explanations for the observed variations concerning the secondary metabolite content of *F. foliacea* and other bryozoans. One is that different secondary metabolite patterns reflect dissimilar ecological or physiological conditions. In this case, secondary metabolite patterns could be interpreted as a reaction of the producing bryozoan to prevailing conditions in its environment (Christophersen, 1991). Another reason could be the presence of different microbial symbionts, probably directly or indirectly involved in the biosynthesis of the compounds. The hypothesis that monoterpenes and probably other secondary metabolites in *F. foliacea* derive from symbionts, e.g., the green microalga *Epicladia flustrae*, was put forward by Anthoni et al. (1990). Our *F. foliacea* samples from Helgoland, however, had no green algal symbionts, and none of the bacteria isolated from our samples produced compounds similar to *Flustra* metabolites (Peters et al., 2003). Yet another possibility for the variation is the existence of genetically different populations, e.g., the existence of closely related but separate species, subspecies, or chemical races.

F. foliacea metabolites may have a specific ecological function. Dihydroflustramine C (**12**) and flustramine D (**18**) showed AHL-antagonistic effects, limited to a certain concentration range (Peters et al., 2003), as have been described for

the halogenated furanones of the red alga *Delisea pulchra* (Givskov et al., 1996; Manefield et al., 1999; Hentzer et al., 2002). Investigations concerned with the localization and surface quantification of secondary metabolites in *D. pulchra* showed that the concentration of halogenated furanones on the surface of the plant is in accordance with furanones functioning as antifouling agents (Dworjanyn et al., 1999). Investigations on the presence of brominated indoles and essential oil components on the surface of *F. foliacea*, and the localization of these metabolites in the tissue of the animal, could answer questions concerning their biological function, their the active concentrations, and probably the alkaloid and terpenoid release mechanisms. The detection of *F. foliacea* metabolites in snails and starfish, obtained from the surface of the bryozoan, could be an indication of the presence of alkaloids outside the animal tissue. Our investigations also indicated that alkaloids may be released into the surrounding water.

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PSEUDOPTEROSIN CONTENT VARIABILITY OF THE PURPLE SEA WHIP *Pseudopterogorgia elisabethae* AT THE ISLANDS OF SAN ANDRES AND PROVIDENCIA (SW CARIBBEAN)

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Abstract—To determine pseudopterosin composition and concentration in colonies of *Pseudopterogorgia elisabethae* from the islands of San Andres and Providencia, we collected fragments of individual colonies at various sites and depth ranges around the islands. Chromatographic profiles of the polar fraction, particularly those obtained by HPLC-MS analyses, allowed us to recognize two different chemotypes. Chemotype 1 characterized samples from Providencia whereas chemotype 2 characterized samples from San Andres. A complex pseudopterosin mixture (compounds **1**–**13**) characterized chemotype 1. These compounds were isolated by a combination of chromatographic methods and identified by spectroscopic methods (MS, UV, ^{1}H, and ^{13}C NMR). We identified the known pseudopterosins G and K and seco-pseudopterosin A. We also isolated and identified seven new compounds, pseudopterosins P-V, isomers of known pseudopterosins. Pseudopterosins G and K were found at concentrations ranging between 1 and 3% of the animal dry mass. Pseudopterosins Q and U were the major compounds reaching up to 6% of the animal dry mass at some locations. Major metabolites in chemotype 2 had a molecular weight and fragmentation pattern different from that observed in the pseudopterosins, as determined by HPLC-MS. Total pseudopterosin concentration in this chemotype was below 3% dry mass at all sites. Total pseudopterosin concentration was significantly higher in chemotype 1, with concentrations ranging between 4 and 20% dry mass. At most locations on Providencia, however, total pseudopterosin concentration ranged between 11 and 15% dry mass. Concentrations exceed reports from other locations in the Caribbean. Furthermore, pseudopterosin composition in our samples is quite different from those in specimens of *P. elisabethae* from the Bahamas and Bermuda. Pseudopterosins G, K, and P-V are characteristic of *P. elisabethae* colonies from the island of Providencia, while pseudopterosins

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A-D are characteristic of colonies of *P. elisabethae* from the Bahamas islands, and pseudopterosins E-L have been isolated from *P. elisabethae* from the Bahamas and Bermuda. The overall morphology of *P. elisabethae* can be variable, and chemical differences are not correlated to specific morphs. We confirmed the species identity of each colony by morphological and sclerite analysis and found no significant differences in sclerite dimensions among different colonies and chemotypes.

Key Words—*Pseudopterogorgia elisabethae*, pseudopterosins, chemical variability, diterpenes, anti-inflammatory activity, Gorgonian, Octocorallia, San Andres and Providencia islands, Colombian Caribbean.

INTRODUCTION

Sea feathers of the genus *Pseudopterogorgia* (Octocorallia: Gorgonacea: Gorgoniidae) are a diverse and common group of soft corals generally thriving in protected reef environments throughout the Caribbean. Diterpenoids, sesquiterpenoids, acetogenins, and steroids isolated from species of *Pseudopterogorgia* have shown potent anti-inflammatory, antitumor, and cytotoxic activity *in vitro* (Fenical, 1987; Rodriguez, 1995). The purple sea feather, *Pseudopterogorgia elisabethae* (Bayer, 1961) in particular, has been a prolific source of diterpenoids with potent *in vivo* anti-inflammatory activity (Look et al., 1986a,b; Roussis et al., 1990), selective cytotoxicity against renal, CNS, and leukemia cancer cell lines (Rodriguez et al., 1998), and *in vitro* activity against *Mycobacterium tuberculosis* (Rodriguez et al., 2000b; Rodríguez and Ramírez, 2001). However, most diterpenoids isolated from *Pseudopterogorgia* species are of interest because they exhibit potent *in vivo* anti-inflammatory activity usually involving unique modes of action (Fenical, 1987; Mayer et al., 1998).

The pseudopterosins are a group of closely related glycosyilated diterpenes originally isolated from Bahamian and Bermudian specimens of the purple sea feather *P. elisabethae* (Look et al., 1986a,b; Roussis et al., 1990). They show superior analgesic activity compared to available anti-inflammatory drugs such as Indomethacin (Look et al., 1986a). They are currently incorporated into several skin care preparations.

The great degree of chemical variation among different specimens of *P. elisabethae* from various sites in the Caribbean region has been acknowledged by several authors. Different kinds of the closely related pseudopterosins have been isolated from Bahamian and Bermudan specimens of *P. elisabethae* (Look et al., 1986a,b; Roussis et al., 1990). The concentrations also vary among sites and between individuals in the same reef (Thornton and Kerr, 2002). In contrast, some collections of *P. elisabethae* from the island of San Andres found pseudopterosins in trace amounts (Rodriguez et al., 1999, 2000a). So far, it is not known if chemical variability and pseudopterosin production are genetically or environmentally determined.

The effectiveness of pseudopterosin-based skin care products has generated a great demand for pseudopterosin-rich extracts of *P. elisabethae* by the chemical industry. This demand has been estimated to be in the order of 30–45 tons per year, but could reach up to 100 tons per year if the resource were made available in other locations of the Caribbean (W. Fenical, 2000). The actual supply of pseudopterosin-rich extracts comes from harvests of *P. elisabethae* populations at the Bahamas islands, but because of great demand for *P. elisabethae* extracts and the complex and expensive syntheses required to produce the pseudopterosins, there is a interest in exploring the potential of this resource in other areas of the Caribbean. To evaluate the potential for regional populations of this species to produce pseudopterosin-rich extracts, we conducted population surveys (Puyana et al., unpublished) and chemical analyses of this species around the islands of Providencia and San Andres, Colombia. The aim of this paper is to document the pseudopterosin composition of specimens of *P. elisabethae* collected in field surveys with particular emphasis on total pseudopterosin content.

METHODS AND MATERIALS

Gorgonian Collection and Identification. This study was performed at the archipelago of San Andres and Providencia (SW Caribbean Sea), which covers an approximate area of 2188 km^2 and includes a series of oceanic islands, cays, and shoals that line the lower Nicaraguan Rise. San Andres and Providencia are the main islands and have significant adjacent recent coralline formations covering an area of 169.6 km^2 (Zea et al., 1998; Diaz et al., 2000). The great geomorphological diversity of the reef complexes at these islands allows the development of various reef habitats with varying depths, ranges of wave action, and kinds of substrate that favor the development of dense and diverse soft coral communities (Diaz et al., 1995; Sanchez et al., 1998).

Fragments of several *P. elisabethae* colonies were collected by SCUBA at various sites and depth ranges around the islands of San Andres and Providencia. Sample collection never involved removing whole colonies, only a terminal fragment of each individual colony was cut from the main gorgonian axis with sharp scissors. Gorgonian fragments were air-dried, labeled, and stored in the freezer. Samples were kept frozen until extraction. For simplicity, we refer to "colony" whenever we refer to a colony fragment.

Colony fragments used for chemical analyses were collected at 13 sites off the island of Providencia and 4 sites off the island of San Andres between June and September 2002 (Figure 1 and Table 1). We collected on average 10 replicates per location. Depth of collection and number of samples collected at each site are listed in Table 1.

The morphology of *P. elisabethae* colonies can be highly variable. Therefore, to avoid confusion with other species, we corroborated the identity of each

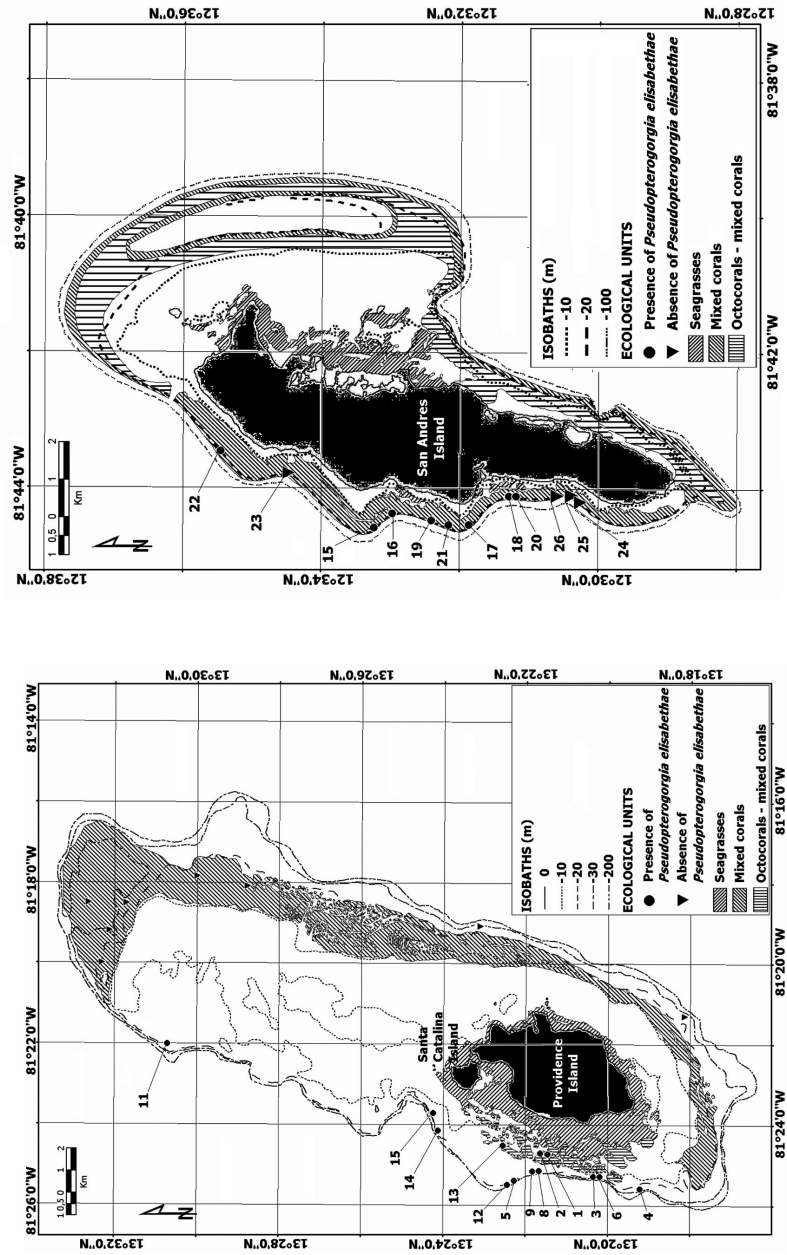

FIG. 1. Location of collecting sites at the islands of Providencia and San Andres.

TABLE 1. DEPTH RANGE (IN METERS) AND TOTAL NUMBER OF SAMPLES (*N*) COLLECTED AT SURVEYED SITES AT SAN ANDRES AND PROVIDENCIA

Site[a]	Depth range (m)	*N*
Providencia Island		
1	13	9
1	23	6
2	13	12
2	23	8
3	17	10
4	20	10
5	23	9
6	17	10
7	18–23	10
8	15–17	10
9	17–27	10
11	25–27	10
12	40	6
13	22–25	9
14	14–20	8
San Andres Island		
15	27–30	11
16	20	10
16	27	10
16	33	11
17	20	18
19	18–23	8

[a] For survey sites, refer to Figure 1.

colony by comparison of the gross colony architecture and by morphology and dimensions of the three most common sclerite types (i.e., spindles, anthocodial rods, and scaphoids). To obtain sclerites, we clipped off a small fragment of the colony and digested it in household bleach. Once the organic tissue was digested, sclerites were repeatedly washed with distilled water and ethanol, and dried in an oven at 70°C for 24 hr. Crude sclerite preparations were mounted on glass slides and analyzed under a microscope. For each colony, we measured 30 anthocodial rods, 30 spindles, and 30 scaphoids. For species designations, we followed Bayer (1961).

Extraction and Isolation of Pseudopterosin Crude Fractions. Each dried colony was cut in small pieces and weighed. One to 2 g of gorgonian tissue were repeatedly extracted with a 1:1 dichloromethane/methanol mixture. Resultant extracts were filtered and concentrated by rotary evaporation obtaining a dark green oily extract. Crude extracts were analyzed by thin layer chromatography (TLC) on silica plates with diethyl ether as the developing solvent. Plates were visualized under UV_{254nm} light followed by charring with 50% H_2SO_4 in ethanol,

using pure pseudopterosin A as a standard on all plates. Characteristic TLC profiles were obtained for each location. A portion of each crude extract was redissolved in methanol for HPLC analysis to obtain a characteristic chemical HPLC profile for each colony. Once we obtained the chemical profile, we identified the pseudopterosins present in the crude extracts. For these analyses, we selected crude extracts that were more representative of each site and chemical profile (based on the higher concentration of some compounds in the crude extracts). Selected extracts were passed through a reverse phase C-18 cartridge and eluted with a 1:1 acetonitrile/water mixture to remove fats, sterols, and pigments. Subsequently, each pseudopterosin crude fraction was analyzed by an HPLC coupled to a Shimadzu QP-8000α Mass Spectrometer. We used APCI ionization mode. Separation was achieved using a Thermo Hypersil-Keystone RP-18 analytical column (100×4 mm i.d., 5 μm), eluting with a water/acetonitrile gradient (30–100%) at a rate of 0.2 ml min^{-1} for 30 min.

Pseudopterosin Isolation and Identification. To identify the pseudopterosins present, we isolated them, using open column chromatography followed by preparative reverse phase HPLC. For open column chromatography, we used normal phase silica gel 60 (0.063–0.200 mm, Merck). Columns were eluted with mixtures of varying proportions of hexane/diclorometh ane, followed by mixtures of ethyl acetate/dichloromethane and ethyl acetate/methanol. Final separation was by preparative reverse phase HPLC with a Merck-Hitachi L-4500 chromatograph coupled to a UV/VIS L-4250 detector. Separation was achieved using a semipreparative Nucleosil RP-18 (300×8 mm i.d., 10 μm) column, eluting with water/acetonitrile gradients (75–100%) at a rate of 2 ml min^{-1}. Compounds were detected at a wavelength of 210 nm. UV spectra for each compound were obtained by using a Merck-Hitachi L-4500 HPLC coupled to a Diode Array detector. Nine pseudopterosins and a seco-pseudopterosin were obtained by HPLC. Each compound was subjected to NMR analysis by using a 400 MHz JEOL Lambda-400 spectrometer with $CDCl_3$ as solvent.

Finally, the isolated compounds were identified by UV, MS, ^1H, and ^{13}C NMR spectroscopy, and by comparison of our spectral data with those previously reported (Look et al., 1986a; Look and Fenical, 1987; Roussis et al., 1990).

Quantification of pseudopterosins in crude extracts was performed by HPLC with UV detection, using a calibration curve generated with pure pseudopterosin A as a standard in various solutions of different concentrations ranging between 0.05 and 3.00 mg ml^{-1}. All known pseudopterosins contain a similar chromophore facilitating their quantification by chromatographic methods using one standard. Aliquots were injected into a Shimadzu SPD-10AVP/10AVVP HPLC, and separation was achieved by using a Dynamax reverse phase C-18 analytical column (250×5 mm i.d., 5 μm) with a water/acetonitrile gradient (30–100%) at a rate of 1 ml min^{-1} for 45 min. Analyses were run in duplicate. We obtained a linear regression corresponding to the equation $y = 3771368.5x - 1173168.3$ ($r^2 = 0.992$).

Quantities of individual and total pseudopterosins were expressed relative to the dry mass of the animal prior to extraction (including gorgonin matrix and coenenchyme).

Statistical Analyses. Statistical analyses were performed by using Statgraphics Plus version 2.0 software. To determine if there were significant differences in the total pseudopterosin concentrations between locations, we used the nonparametric Kruskall–Wallis test, given that we were unable to normalize data despite various transformations (square root or arcsin). Quantities of individual pseudopterosins were averaged for each site and analyzed.

RESULTS

Chemical Profiles. TLC analyses showed important differences between extracts from samples collected at the island of Providencia and extracts from samples collected at San Andres. The overall morphology of *P. elisabethae* can be variable, and chemical differences were not correlated to specific morphs. We confirmed species identity of each colony by morphological and sclerite analysis and found no significant differences in sclerite dimensions among different colonies. Therefore, we ruled out the possibility that we were dealing with two different species.

HPLC profiles of crude extracts of *P. elisabethae* (Figure 2) allowed us to confirm chemical differences among colonies of *P. elisabethae* collected at Providencia and San Andres. These analyses allowed us to determine two distinct chemotypes. Chemotype 1 (Figure 2a) is characteristic and exclusive of samples collected at Providencia. Chemotype 2 (Figure 2b) is characteristic but not exclusive to samples collected at San Andres. Occasionally we found chemotype 2 in samples from Providencia, but never found chemotype 1 in samples collected at San Andres. Although quantities of individual metabolites varied and some minor metabolites were absent in some cases, the general secondary metabolite profiles were consistent for each chemotype.

HPLC-MS analyses allowed us to identify compounds **1**–**13** as pseudopterosins. They were present in samples of both chemotypes. In all cases, besides the pseudomolecular weight ion, the most abundant fragment was m/z 301, corresponding to the $[\text{aglycon}+\text{H}]^+$, followed by the fragment originating from the loss of isopropylidene unit from the aglycone. Chemotype 1 is rich in pseudopterosins and a seco-pseudopterosin (compounds **1**–**13**), pseudopterosin Q being the most abundant compound in all extracts. In contrast, chemotype 2 provided only minor concentrations of pseudopterosins (below 3% dry mass). Since, chemotype 1 was found only in samples collected at Providencia, we concentrated our efforts in those samples.

After isolation, identity of the different pseudopterosins was determined by MS and NMR spectroscopy. Structural elucidation of the new pseudopterosins

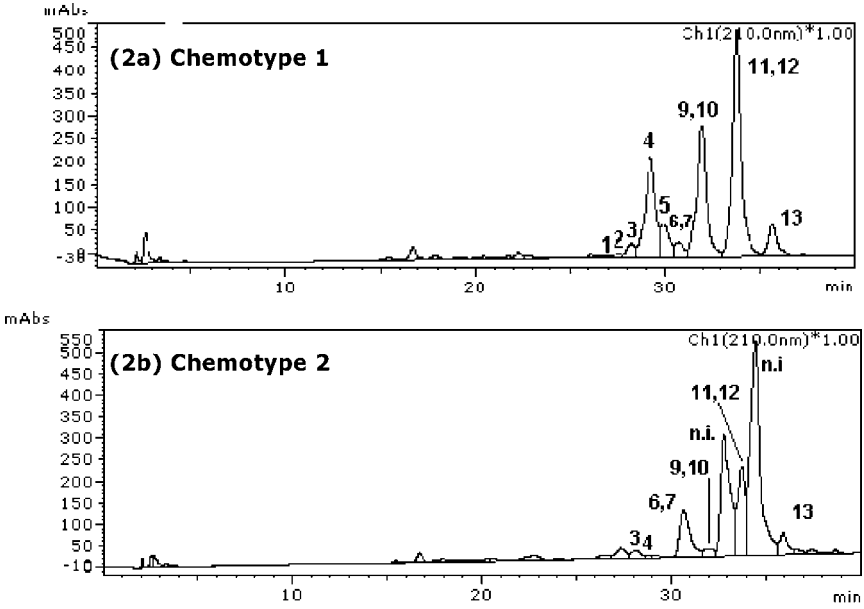

FIG. 2. (a,b). Chromatographic profiles of the two chemotypes found in samples of *Pseudopterogorgia elisabethae* collected at San Andres and Providencia. Compounds **1**–**13** are pseudopterosins (n.i. = not identified).

and their complete stereochemistry will be published later (Duque et al., unpublished). The identities are listed in Table 2. Structures are shown in Figure 3.

Quantification of Pseudopterosins. We analyzed 13 sites at Providencia ($N = 137$ colonies) and 4 sites at San Andres ($N = 67$ colonies). In some cases, we collected samples from different depths at the same site (sites 1 and 2 at Providencia and site 16 at San Andres). For statistical analyses, however, different depth ranges at the same location were considered as separate locations. The concentration of individual and total pseudopterosins for both islands is shown in Figure 4, and the concentration of pseudopterosins (compounds **3**–**12**) for samples from Providencia and for samples from San Andres appear in Tables 3–5, respectively.

Statistical Analyses. Given the obvious differences between chemotypes and pseudopterosin concentrations on the islands of San Andres and Providencia, we compared compound concentrations among locations for each island. For Providencia, there were significant differences in the concentration of compounds **2**–**8** and total pseudopterosin concentration among sites ($\alpha = 0.05$). In all cases, the calculated value was greater than the critical value (Table 6).

TABLE 2. IDENTIFICATION OF PSEUDOPTEROSINS IN CHEMOTYPES 1 AND 2 FROM SAMPLES OF *Pseudopterogorgia elisabethae* COLLECTED AT SAN ANDRES AND PROVIDENCIA

Compound[a]	Identity	R^b_{RT}	Molecular weight[c]	Relative %[d]
1	Unidentified	1.00	432	1.3
2	Unidentified mixture of seco-pseudopterosins and pseudopterosins	1.03	432–434	2.4
3	Pseudopterosin T	1.06	432	7.4
4	Pseudopterosin K	1.09	446	11.7
5	Pseudopterosin G	1.13	446	5.8
6	Pseudopterosin P	115	446	11.1[e]
7	*seco*-Pseudopterosin		434	
8[f]	Unidentified mixture of seco-pseudopterosins and	—	448–474	—
9	Pseudopterosin U	1.21[g]	474	19.3
10	Pseudopterosin V		474	6.5
11	Pseudopterosin Q	1.27[h]	488	20.0
12	Pseudopterosin R		488	6.7
13	Pseudopterosin S	1.35	488	7.8

[a] For compound numbers, refer to Figure 2a,b.
[b] Relative retention time compared to pseudopterosin A standard.
[c] As determined by HPLC-MS in APCI ionization mode.
[d] Relative percentage compared to total pseudopterosin content in all samples.
[e] Initially isolated as a single compound.
[f] Not quantified.
[g] Compounds **9** and **10**.
[h] Compounds **11** and 12 were originally isolated as a single peak in each case, further separation allowed us to calculate the proportion between the two compounds in each mixture.

At San Andres, however, we only detected significant differences for compound **6** ($\alpha = 0.05$, 5 df) ($H_{\text{calc}} = 18.06$, $H_{\text{crit}} = 11.07$). For discussion purposes, we consider only total pseudopterosin concentration. Total pseudopterosin concentration was greater at Providencia than at San Andres, but with wide variation among sites. Low values of total pseudopterosin concentration correspond to those sites where we found chemotype 2 (sites 5, 7, 8, 11, 12). However, samples of chemotype 2 from Providencia always had a greater total pseudopterosin concentration when compared to samples from San Andres. We grouped sites on the basis of total pseudopterosin concentration. Sites 13 and 14 had a total pseudopterosin concentration between 15 and 20% dry mass, sites 1, 2, 3, 4, and 6 had a total pseudopterosin concentration between 11 and 15% dry mass, and sites 5, 7, 8, 11, and

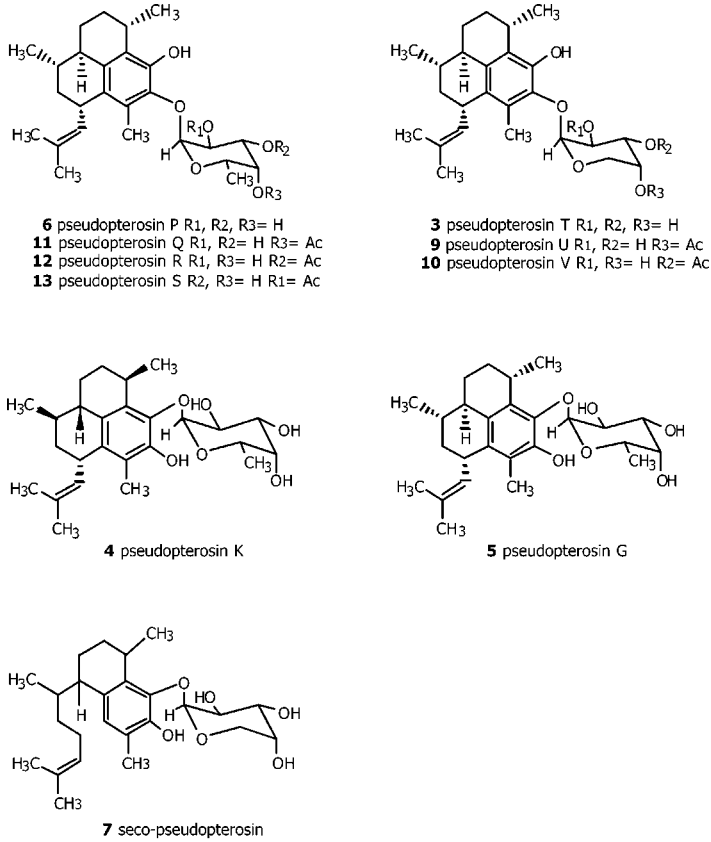

FIG. 3. Structures of new and known pseudopterosins and a seco-pseudopterosin isolated and identified from samples of *Pseudopterogorgia elisabethae* from the islands of San Andres and Providencia.

12 were grouped because total pseudopterosin concentration ranged between 4 and 6% dry mass. Sites 15, 16, 17, and 19 at San Andres Island always had a total pseudopterosin concentration below 3% dry mass. These results are shown in Figure 4.

DISCUSSION

We found qualitative and quantitative differences in crude extracts of *P. elisabethae* from San Andres and Providencia. Samples from the island of Providencia had a greater number of compounds when compared with samples from San Andres. Additionally, major compounds in extracts from both islands were not the same. Major compounds collected at Providencia were pseudopterosins, while

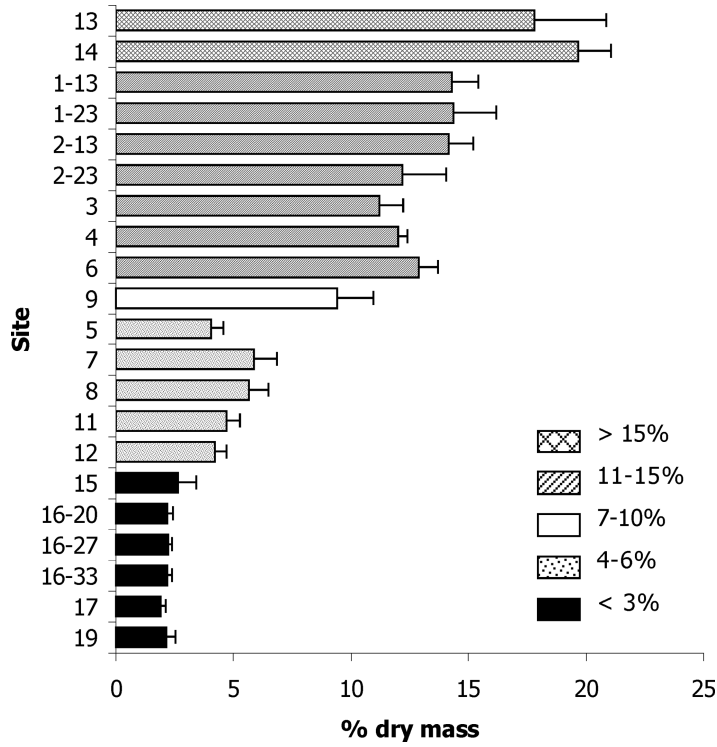

FIG. 4. Grouping of sites on the basis of the concentration of total pseudopterosins (mean ± standard error) in samples of *Pseudopterogorgia elisabethae* collected at Providencia and San Andres. Concentration expressed as percentage of animal dry mass. Numbers after sites indicate depth ranges within sites.

major compounds in samples from San Andres were not. We found pseudopterosins in samples from San Andres, but in a much lower concentration compared to samples from Providencia.

Thus, we established two chemotypes. Chemotype 1 was characterized by a complex mixture of pseudopterosins and seco-pseudopterosins (compounds **1**–**13**), with compounds **9** and **10** (pseudopterosins Q and U) being the major components. This chemotype was unique in samples from Providencia. Chemotype 2 on the other hand was characterized by having low concentrations of pseudopterosins, and its major compounds were not of the pseudopterosin class. Chemotype 2 was characteristic of samples collected at San Andres but was also found at some locations at Providencia (sites 5, 7, and 8). Additionally, we detected both chemotypes in samples collected at site 9. In summary, at Providencia Island we detected

TABLE 3. MEAN (X_o)± STANDARD ERROR (SE) AND RANGE OF INDIVIDUAL AND TOTAL PSEUDOPTEROSIN CONCENTRATION IN SAMPLES OF *Pseudopterogorgia elisabethae* COLLECTED AT SITES 1–6 AT PROVIDENCIA

		Site							
Compound		1 (13 m), $N = 9$	1 (23 m), $N = 6$	2 (13 m), $N = 12$	2 (23 m), $N = 8$	3 (17 m), $N = 10$	4 (20 m), $N = 10$	5 (23 m), $N = 9$	6 (17 m), $N = 10$
3	X ± SE	1.1 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	0.8 ± 0.03	0.6 ± 0.1	1.0 ± 0.1
	Range	0.7–1.7	0.5–1.3	0.6–1.6	0.4–1.5	0.4–1.2	0.6–0.9	0.4–1.6	0.5–1.5
4	X ± SE	2.1 ± 0.3	2.0 ± 0.3	2.1 ± 0.2	1.6 ± 0.3	1.8 ± 0.2	1.9 ± 0.1	0.2 ± 0.1	2.1 ± 0.2
	Range	0.8–3.6	0.8–3.5	1.0–3.2	0.8–3.0	1.2–3.2	1.5–2.2	0.0–0.7	1.1–3.4
5	X ± SE	1.3 ± 0.1	1.1 ± 0.2	1.2 ± 0.1	1.1 ± 0.2	0.8 ± 0.1	1.0 ± 0.05		1.3 ± 0.1
	Range	0.7–1.8	0.5–1.8	0.6–2.2	0.6–2.0	0.0–1.2	0.8–1.6		0.6–2.2
6 and **7**	X ± SE	0.8 ± 0.2	0.6 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.04	0.9 ± 0.1	0.8 ± 0.1
	Range	0.0–1.5	0.0–1.0	0.6–1.5	0.0–1.1	0.4–1.0	0.5–1.0	0.5–1.6	0.6–1.3
9 and **10**	X ± SE	4.4 ± 0.4	4.8 ± 0.7	4.2 ± 0.3	3.8 ± 0.7	3.4 ± 0.4	3.3 ± 0.2	0.7 ± 0.1	3.5 ± 0.3
	Range	2.5–6.4	2.2–6.6	2.2–5.7	1.5–6.8	1.3–5.5	2.5–4.1	0.4–1.5	1.5–4.9
11 and 12	X ± SE	3.8 ± 0.4	3.6 ± 0.5	4.0 ± 0.3	3.4 ± 0.5	3.0 ± 0.4	3.4 ± 0.1	1.2 ± 0.2	3.3 ± 0.3
	Range	2.1–6.3	1.6–5.4	2.3–5.4	1.6–5.6	0.4–4.3	2.9–4.4	0.7–2.2	2.2–4.9
13	X ± SE	0.9 ± 0.1	1.2 ± 0.1	1.0 ± 0.05	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.04	0.4 ± 0.05	0.9 ± 0.05
	Range	0.6–1.5	0.8–1.6	0.7–1.2	0.4–1.3	0.3–1.2	0.6–1.0	0.3–0.8	0.6–1.0
Total	X ± SE	14.3 ± 1.1	14.4 ± 1.8	14.1 ± 1.0	12.2 ± 1.8	11.2 ± 1.0	12.0 ± 0.4	4.0 ± 0.5	12.9 ± 0.8
	Range	9.5–21.8	7.0–20.4	8.5–20.1	6.0–21.3	4.9–15.2	10.0–13.7	2.8–7.7	9.5–15.8

Note. Quantities expressed as percentage of compound per dry mass of the animal prior to extraction. Numbers in parenthesis indicate depth of collection in meters. *N* indicates the number of samples analyzed per site. Compounds **6** and **7**, **9** and **10**, and **11** and **12** were originally isolated as a single peak in each case and quantified as such.

TABLE 4. MEAN (X_o) ± STANDARD ERROR (SE) AND RANGE OF INDIVIDUAL AND TOTAL PSEUDOPTEROSIN CONCENTRATION IN SAMPLES OF *Pseudopterogorgia elisabethae* COLLECTED AT SITES 7–14 AT PROVIDENCIA

		Site						
Compound		7 (18–23 m), $N = 10$	8 (15–17 m), $N = 10$	9 (17–27 m), $N = 10$	11 (25–27 m), $N = 10$	12 (40 m), $N = 6$	13 (22–25 m), $N = 9$	14 (14–20 m), $N = 8$
3	X ± SE	0.5 ± 0.2	0.3 ± 0.1	0.6 ± 0.2	0.5 ± 0.04	0.4 ± 0.05	1.2 ± 0.2	0.9 ± 0.03
	Range	0.0–1.4	0.0–0.6	0.0–1.5	0.3–0.8	0.2–0.5	0.7–3.2	0.8–1.1
4	X ± SE	0.3 ± 0.1	0.5 ± 0.2	1.2 ± 0.5	0.4 ± 0.1	0.4 ± 0.1	2.2 ± 0.3	3.2 ± 0.3
	Range	0.0–0.6	0.0–2.4	0.0–4.0	0.0–0.8	0.0–0.7	1.3–5.0	1.9–4.4
5	X ± SE	0.1 ± 0.1		0.7 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	1.2 ± 0.2	1.4 ± 0.1
	Range	0.0–0.4		0.0–2.0	0.0–0.7	0.0–0.4	0.6–3.5	1.0–1.8
6 and **7**	X ± SE	1.0 ± 0.2	1.2 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.0 ± 0.2	1.2 ± 0.2	1.3 ± 0.2
	Range	0.3–2.3	0.9–2.4	0.8–1.8	0.8–2.0	0.7–1.8	0.7–3.0	0.7–1.9
9 and **10**	X ± SE	2.0 ± 0.7	1.1 ± 0.2	2.3 ± 0.5	0.8 ± 0.1	0.7 ± 0.1	5.9 ± 0.9	5.3 ± 0.5
	Range	0.6–6.2	0.5–2.8	0.7–5.7	0.4–1.5	0.4–0.9	3.3–15.0	2.6–7.8
11 and **12**	X ± SE	1.1 ± 0.1	2.0 ± 0.3	2.6 ± 0.3	1.3 ± 0.2	1.2 ± 0.2	4.8 ± 0.9	6.2 ± 0.4
	Range	0.6–1.5	1.2–4.0	1.2–4.3	0.6–3.0	0.3–2.0	2.9–13.6	5.3–8.6
13	X ± SE	0.9 ± 0.3	0.5 ± 0.05	0.6 ± 0.06	0.4 ± 0.03	0.3 ± 0.04	1.3 ± 0.2	1.4 ± 0.2
	Range	0.3–3.9	0.4–0.9	0.4–0.9	0.3–0.6	0.1–0.4	0.9–3.6	0.6–2.2
Total	X ± SE	5.9 ± 1.0	5.6 ± 0.9	9.4 ± 1.6	4.7 ± 0.6	4.2 ± 0.5	17.8 ± 3.0	19.7 ± 1.4
	Range	3.1–11.7	3.6–12.5	3.9–16.3	2.9–9.3	2.2–5.8	11.4–47	13.9–27.1

Note. Quantities expressed as percentage of compound per dry mass of the animal prior to extraction. Numbers in parenthesis indicate depth of collection in meters. *N* indicates the number of samples analyzed per site. Compounds **6** and **7**, **9** and **10**, and **11** and **12** were originally isolated as a single peak in each case and quantified as such.

TABLE 5. MEAN (X) ± STANDARD ERROR (SE) AND RANGE OF INDIVIDUAL AND TOTAL PSEUDOPTEROSIN CONCENTRATION IN SAMPLES OF *Pseudopterogorgia elisabethae* COLLECTED AT SITES 15–19 AT SAN ANDRES

		Site					
Compound		15 (27–30 m), $N = 10$	16 (20 m), $N = 10$	16 (27 m), $N = 10$	16 (33 m), $N = 10$	17 (18–20 m), $N = 6$	19 (18–23 m), $N = 9$
3	X ± SE	0.1 ± 0.04	0.05 ± 0.02	0.1 ± 0.02	0.05 ± 0.01	0.07 ± 0.01	0.09 ± 0.01
	Range	0.02–0.4	0.0–0.2	0.05–0.2	0.0–0.1	0.0–0.2	0.03–0.1
4	X ± SE	0.04 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.05 ± 0.01	0.04 ± 0.01
	Range	0.0–0.06	0.0–0.1	0.0–0.1	0.0–0.05	0.0–0.2	0.01–0.1
5	X ± SE						
	Range						
6 and **7**	X ± SE	0.4 ± 0.2	0.3 ± 0.1	0.4 ± 0.1	0.2 ± 0.05	0.2 ± 0.03	0.2 ± 0.1
	Range	0.03–2.5	0.0–1.1	0.1–1.1	0.06–0.5	0.0–0.4	0.03–0.5
9 and **10**	X ± SE	0.2 ± 0.1	0.04 ± 0.04	0.2 ± 0.03	0.1 ± 0.01	0.1 ± 0.02	0.1 ± 0.03
	Range	0.0–1.1	0.0–0.4	0.0–0.2	0.04–0.2	0.0–0.4	0.02–0.3
11 and **12**	X ± SE	1.8 ± 0.5	1.6 ± 0.2	1.3 ± 0.1	1.6 ± 0.2	1.4 ± 0.1	1.5 ± 0.2
	Range	0.4–6.8	0.3–2.1	0.9–1.5	0.6–2.4	0.7–2.5	0.4–2.6
13	X ± SE	0.2 ± 0.06	0.1 ± 0.03	0.2 ± 0.04	0.2 ± 0.05	0.1 ± 0.03	0.1 ± 0.04
	Range	0.0–0.6	0.02–0.4	0.04–0.4	0.03–0.6	0.04–0.4	0.01–0.3
Total	X ± SE	2.6 ± 0.8	2.2 ± 0.3	2.2 ± 0.2	2.2 ± 0.2	1.9 ± 0.2	2.1 ± 0.4
	Range	0.7–9.9	0.4–3.6	1.6–3.5	1.1–3.3	1.0–3.5	0.6–4.0

Note. Quantities expressed as percentage of compound per dry mass of the animal prior to extraction. Numbers in parenthesis indicate depth of collection in meters. N indicates the number of samples analyzed per site. Compounds **6** and **7**, **9** and **10**, and **11** and **12** were originally isolated as a single peak in each case and quantified as such.

TABLE 6. RESULTS OF KRUSKALL–WALLIS TEST FOR COMPARISON OF TOTAL AND INDIVIDUAL PSEUDOPTEROSIN CONCENTRATION BETWEEN SITES AT PROVIDENCIA

Compound[a]	H_{calc}	H_{crit}
2	41.02	23.68
3	63.62	23.68
4	66.30	23.68
5	28.41	23.68
6	67.69	23.68
7	70.35	23.68
8	88.05	23.68
Total	67.85	23.68

[a] H_{calc}, calculated value; H_{crit}, chi-square table, 14 df, and $\alpha = 0.05$.

chemotypes 1 and 2, whereas chemotype 2 characterized all samples collected at San Andres Island.

We found significant differences in the concentrations of individual and total pseudopterosins in samples collected at different sites at Providencia. Total pseudopterosin concentration was greater in samples collected at Providencia Island compared to those at San Andres Island. At Providencia Island, however, concentration ranges were quite variable among sites. Sites 13 and 14 were those in which we found higher total pseudopterosin concentration (17.8 and 19.7% dry mass, respectively). At several sites (5, 6, 7, 8, 11, and 12) however, total pseudopterosin concentration was found to be in the range between 4 and 6% dry mass. These low values correspond to sites where we also found colonies of chemotype 2. Total pseudopterosin concentrations in samples from San Andres Island were found to be less than 3% dry mass.

Our results are interesting when compared to published reports from other Caribbean locations. Total pseudopterosin concentration in *P. elisabethae* from the Bahamas islands has been reported to be around 8% dry mass (Look et al., 1986a). Our findings show that at some sites in Providencia Island, total pseudopterosin concentration can be two to three times higher. However, pseudopterosin composition is highly variable depending on location. Pseudopterosins A-D are characteristic in most Bahamian specimens of *P. elisabethae*, with pseudopterosin C usually being the major compound (Look et al., 1986b; Thornton and Kerr, 2002). Roussis et al. (1990), isolated pseudopterosins E-J from *P. elisabethae* samples collected at Bermuda, where pseudopterosins E and F were the major constituents of polar fractions of the extract. The authors also isolated pseudopterosins K and L from samples collected at Great Abaco Island, Bahamas.

In this study, we confirmed the presence of seco-pseudopterosins in samples of *P. elisabethae*. The seco-pseudopterosins exhibit high anti-inflammatory activity (Look and Fenical, 1987). Thornton and Kerr (2002) used TLC analyses to corroborate the identity of *P. elisabethae* colonies collected in the Bahamas. They recognized *P. elisabethae* as such by the presence of a characteristic pseudopterosin band. In our case, however, TLC analyses were not sufficient to corroborate species designations. We found a high degree of pseudopterosin variability between sites and even among colonies collected at the same site. This was easily observed in the HPLC-MS profiles that were obtained from each extract.

Chemical diversity in soft corals may have an adaptive value preventing predation and mediating ecological interactions in reef environments (Sammarco and Coll, 1988). Several studies have demonstrated that interactions with other organisms such as predation (Pawlik et al., 1987; Puglisi et al., 2000; Thornton and Kerr, 2002), competitive and allelopathic interactions (De Nys et al., 1991; Maida et al., 1993, 1995; Leone et al., 1995), and reproductive state (Coll et al., 1989, 1995) may influence the kind and quantities of specific compounds produced by soft corals. When trying to understand chemical variability, the issue is complicated by the influence of ecological variables such as habitat characteristics and depth (Harvell et al., 1993; Roussis et al., 2000; Thornton and Kerr, 2002). At this point, we do not know the causes for the extreme chemical variability of *P. elisabethae* in the areas we surveyed.

Thornton and Kerr (2002) were successful in determining some inducing mechanisms for pseudopterosin production in *P. elisabethae* at Grand Bahama Island. They found that predation by the ovulid gastropod *Cyphoma gibbossum*, or decreased levels of UV radiation had a significant effect on the increase of pseudopterosins. On the other hand, simulated wounding or predation by the butterfly fish *Chaetodon capistratus* did not have an effect on the overall concentration of pseudopterosins. Interestingly, the greatest increase of pseudopterosin production occurred in response to decreased UV radiation. The authors argue that pseudopterosin biosynthesis may involve, directly or indirectly, a photosynthetic organism (probably zooxanthellae). A great diversity of zooxanthellae has been recognized in hermatypic corals (Rowan and Knowlton, 1995; Toller et al., 2001). This diversity is closely related to environmental variables and can change in response to environmental stress. *P. elisabethae* harbors zooxanthellae (Rowan, 1988), and one would expect to find different symbionts on a wide geographical range (i.e., the Caribbean). If zooxanthellae were somehow involved in the biosynthesis of pseudopterosins or other metabolites that would explain, at least partly, the great chemical variation found in *P. elisabethae* among the sites studied so far. There is still controversy, however, regarding the involvement of associated zooxanthellae in pseudopterosin biosynthesis.

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LIGNIFICATION AND RELATED ENZYMES IN *Glycine max* ROOT GROWTH-INHIBITION BY FERULIC ACID

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Abstract—Changes in soluble and cell wall bound peroxidase (POD, EC 1.11.1.7) activity, phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) activity, and lignin content in roots of ferulic acid-stressed soybean (*Glycine max* (L.) Merr.) seedlings and their relationships with root growth were investigated. Three-day-old soybean seedlings were cultivated in half-strength Hoagland nutrient solution containing 1.0 mM ferulic acid for 24–72 hr. Length, fresh weight, and dry weight of roots decreased, while soluble and cell wall bound POD activity, PAL activity, and lignin content increased after ferulic acid treatment. These enzymes probably participate in root growth reduction in association with cell wall stiffening related to the formation of cross-linking among cell wall polymers and lignin production.

Key Words—Allelopathy, ferulic acid, lignin, peroxidase, phenylalanine ammonia-lyase, root growth, soybean.

INTRODUCTION

Plants release organic compounds into the environment from their aerial or subaerial parts as exudates, volatiles, and/or decomposition residues. Released chemicals are accumulated in the soil environment and can affect the growth and development of neighboring plants, an interaction called allelopathy. Among different types of allelopathic agents, phenylpropanoid pathway metabolites have been shown to inhibit seed germination, and reduce root and plant growth (Siqueira et al., 1991; Einhellig, 1995; Macias, 1995).

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Ferulic acid (FA), a phenylpropanoid metabolite, is a strong allelochemical that is frequently found in soil extracts. FA affects plant growth at concentrations of up to 10 mM (Kuiters, 1990; Macias, 1995). Exposure of plant roots to FA reduces water utilization (Holappa and Blum, 1991), inhibits foliar expansion (Blum and Rebbeck, 1989) and root elongation (Kobza and Einhellig, 1987; Baleroni et al., 2000; Pramanik et al., 2000; Ng et al., 2003), and decreases nutrient uptake (Lyu and Blum, 1990; Bergmark et al., 1992; Booker et al., 1992). Further, this allelochemical rapidly depolarizes root cell membranes causing a generalized increase in membrane permeability, inducing lipid peroxidation, and affecting certain enzymatic activities (Baziramakenga et al., 1995; Devi and Prasad, 1996; Politycka, 1998). FA may be esterified with cell wall polysaccharides, incorporated into lignin structures, or form bridges that connect lignin with wall polysaccharides. Rigidity of cell walls and restriction of cell growth are the result (Iiyama et al., 1990; Sánchez et al., 1996).

Although the effects of FA on several physiological events in plants have been studied, less effort has been spent investigating soybean (*Glycine max* (L.) Merr.) roots (Einhellig and Eckrich, 1984; Baziramakenga et al., 1995; Doblinski et al., 2003). Since knowledge of FA's mechanism of action in soybean is scanty, the manner FA affects POD and PAL activities, lignin content, and root growth in seedlings was analyzed.

METHODS AND MATERIALS

General Procedures. Soybean (*Glycine max* (L.) Merr. *cv.* BRS-133) seeds, surface-sterilized with 2% sodium hypochlorite for 2 min and rinsed extensively with deionized water, were dark-germinated (at 25°C) on two sheets of moistened filter paper. Twenty-five, 3-day-old seedlings of uniform size were supported on an adjustable acrylic plate and transferred to a glass container (10 × 16 cm) filled with 200 ml of half-strength Hoagland's solution (pH 6.0) with or without 1.0 mM FA (Ferrarese et al., 2000a). The container was kept in a growth chamber (25°C, 12L:12D photoperiod, irradiance of 280 μmol m^{-2} s^{-1}). The nutrient solution was aerated continuously by air bubbling. Roots were exposed to the allelochemical for 24–72 hr, and the nutrient solution was completely renewed every 24 hr. All roots were measured at the start and end of experiments. Root fresh weight (FW) was determined immediately after incubation and dry weight (DW) estimated after oven drying at 80°C for 24 hr. FA was purchased from Sigma Chemical Co. (St. Louis, USA) and all other reagents used were of chromatographic grade or the purest grade available.

Enzymatic Activities. After incubation, all treated or untreated seedling roots were detached and enzymes were extracted. Peroxidase (POD) was extracted from fresh roots (0.5 g) with 67 mM phosphate buffer (5 ml, pH 7.0). The extract was centrifuged (10,000*g*, 15 min, 4°C), and the supernatant was used to determine the

activity of soluble POD. To isolate cell wall bound POD, the pellet was washed with deionized water until no soluble POD activity was detected in the supernatant. The pellet was incubated in 1 M NaCl (4 ml, 4°C, 60 min), and the homogenate was centrifuged (10,000*g* for 15 min). The supernatant was considered to contain the cell wall-(ionically)-bound POD. Guaiacol-dependent activities of the soluble and cell wall-bound POD were determined according to Cakmak and Horst (1991), with modifications (Herrig et al., 2002). The reaction mixture contained 25 mM sodium phosphate buffer (3 ml, pH 6.8), 2.58 mM guaiacol, and 10 mM H_2O_2. The reaction was initiated by addition of enzyme extract in phosphate buffer. Guaiacol oxidation was followed for 5 min at 470 nm, and enzyme activity was calculated from the extinction coefficient (25.5 mM^{-1} cm^{-1}) for tetraguaiacol. The blank consisted of a reaction mixture without enzyme extract whose absorbance was subtracted from the mixture with enzyme extract. POD activities were expressed as μmol tetraguaiacol min^{-1} g^{-1} FW.

Phenylalanine ammonia-lyase (PAL) was extracted as described by Ferrarese et al. (2000b). Fresh roots (2 g) were ground at 4°C in 0.2 M sodium borate buffer (pH 8.8). Homogenates were centrifuged (12,000*g* for 15 min), and the supernatant was used as the enzyme preparation. The reaction mixture (100 μmoles sodium borate buffer, pH 8.7, and a suitable amount of enzyme extract in a final volume of 1.55 ml) was incubated at 40°C for 5 min for the PAL activity assay. Fifteen μmoles of L-phenylalanine were added to start the reaction. The reaction was stopped after 1 hr of incubation by addition of 50 μl 5 N HCl. Samples were filtered through a 0.45 μm disposable syringe filter (Hamilton® Co., Nevada, USA) and analyzed (20 μl) with a Shimadzu® Liquid Chromatograph (Tokyo, Japan) equipped with a LC-10AD pump, a Rheodine® injector, a SPD-10A UV detector, a CBM-101 Communications Bus Module, and a Class-CR10 workstation system. A reversed-phase Shimpack® GLC-ODS (M) column (150 × 4.6 mm, 5 μm) was used at room temperature together with an equivalent precolumn (10 × 4.6 mm). The mobile phase was methanol: water (70%:30%) with a flow rate of 0.5 ml min^{-1}. Absorbance was measured at 275 nm. Data collection and integration were performed with the Class-CR10 software (Shimadzu®, Tokyo, Japan). *t*-Cinnamate, the product of PAL, was identified by retention time. Parallel controls without L-phenylalanine or with *t*-cinnamate (added as internal standard in the reaction mixture) were as described by Ferrarese et al. (2000b). PAL activity was expressed as nmol *t*-cinnamate min^{-1} g^{-1} of FW.

Lignin Quantification. After the incubation period, dry roots (0.3 g) were homogenized in 50 mM potassium phosphate buffer (7 ml pH, 7.0) with mortar and pestle, and transferred into a centrifuge tube (Ferrarese et al., 2002). The pellet was centrifuged (1,400*g*, 4 min) and washed by successive stirring and centrifugation, as follows: twice with phosphate buffer pH 7.0 (7 ml); × 3 with 1% (v/v) Triton® X-100 in pH 7.0 buffer (7 ml); × 2 with 1 M NaCl in pH 7.0 buffer

(7 ml); × 2 with distilled water (7 ml); and × 2 with acetone (5 ml). The pellet was dried in an oven (60°C for 24 hr) and cooled in a vacuum desiccator. The dry matter obtained was defined as the protein-free cell wall fraction. Further, dry protein-free tissue (0.1 g) was placed into a screw-cap centrifuge tube containing the reaction mixture (1.2 ml of thioglycolic acid plus 6 ml of 2 M HCl) and heated (95°C, 4 hr). After cooling at room temperature, the sample was centrifuged (1,400*g*, 5 min) and the supernatant decanted. The pellet was washed × 3 with distilled water (7 ml), and the reaction product was extracted by shaking (30°C, 18 hr, 115 oscillations min^{-1}) in 0.5 M NaOH (6 ml). After centrifugation (1,400*g*, 5 min), the supernatant was stored and mixed with the supernatant obtained from a second pellet washed in 0.5 M NaOH (3 ml). The combined alkali extracts were acidified with concentrated HCl (1.8 ml). The lignothioglycolic acid (LTGA) formed after 4 hr at 0°C was recovered by centrifugation (1,400*g*, 5 min) and washed × 2 with distilled water (7 ml). The pellet was dried at 60°C, dissolved in 0.5 M NaOH, and diluted to yield an appropriate absorbance for spectrophotometric determination at 280 nm.

Statistical Analyses. Data from 4–8 separate experiments were combined. Statistical tests were performed with *InStat*® (Version 1.12a, GraphPAD Software, San Diego, USA). Statistical significance of the difference between parameters was evaluated by ANOVA ($P < 0.05$).

RESULTS

Ferulic acid decreases primary root lengths at all incubation times (Table 1). Root length was reduced by 71.3% after 24 hr of FA treatment compared to control

TABLE 1. CHANGES IN ROOT LENGTH, ROOT FRESH WEIGHT, AND ROOT DRY WEIGHT OF SOYBEAN SEEDLINGS TREATED WITH 1.0 MM FA

Variable	Treatment	24 hr	Inhibition (%)	48 hr	Inhibitio (%)	72 hr	Inhibition (%)
Root length (cm)	Control	3.34 ± 0.211		4.99 ± 0.229		10.3 ± 0.552	
	FA	0.96 ± 0.065*	71.3	1.49 ± 0.059*	70.1	3.19 ± 0.250*	69.0
Fresh weight (g)	Control	2.52 ± 0.060		2.98 ± 0.048		5.31 ± 0.094	
	FA	1.93 ± 0.035*	23.4	2.03 ± 0.022*	31.9	3.03 ± 0.127*	42.9
Dry weight (g)	Control	0.17 ± 0.004		0.20 ± 0.048		0.33 ± 0.002	
	FA	0.14 ± 0.03^{ns}	20.0	0.15 ± 0.002*	26.7	0.24 ± 0.004*	26.7

Note. *Values are significantly different at the 0.05 level by ANOVA. ns = not significant at 0.05 level.

roots and remained at that level for all experimental treatment times. Likewise, root FW and DW also decreased in contrast to control root. FW gains diminished from 23.4 to 42.9% under the influence of FA and root DW was 26.7% less than control roots at 48 and 72 hr.

Seedlings cultivated with 1.0 mM FA increased in soluble POD activity following treatment (Figure 1A), with significant elevation over the controls after 24 hr (30%), 48 hr (108%), and 72 hr (71%). FA stimulated cell wall-bound POD activity by 29% at 24 hr, 112% at 48 hr, and 156% after 72 hr (Figure 1B). Similarly, FA-affected PAL activities were significantly different from controls (Figure 2). FA increased the enzymatic activities by 55, 63, and 52% after 24, 48, and 72 hr of treatment, respectively.

Lignin content in soybean roots increased following FA treatment by 42, 39, and 61%, at 24, 48, and 72 hr, respectively (Figure 3).

DISCUSSION

Root growth (root length, FW, and DW) decreased with FA exposure (Table 1), while POD and PAL activities and lignin content increased (Figures 1–3). Similar changes in root growth of plants treated with FA have been reported. For example, the hypocotyl length of mung bean (*Phaseolus aureus* L.) decreased by about 40% after 1.0 mM FA treatment (Demos et al., 1975). Vaughan and Ord (1990) reported that root length and FW of pea (*Pisum sativum* L.) was significantly inhibited by 1.0 mM FA (>70%). Exogenous application of 1.0 mM FA decreases the growth

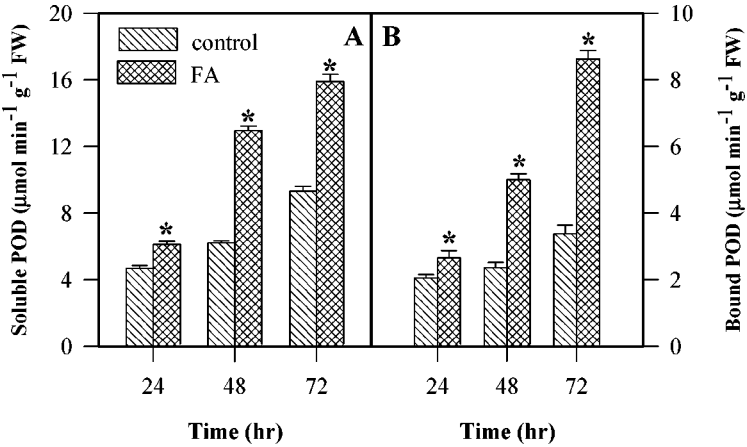

FIG. 1. Effects of FA on soluble (A) and cell wall-bound (B) peroxidases. *Values ($N = 4$ to 8 ± SEM) differing significantly from the control ($P < 0.05$).

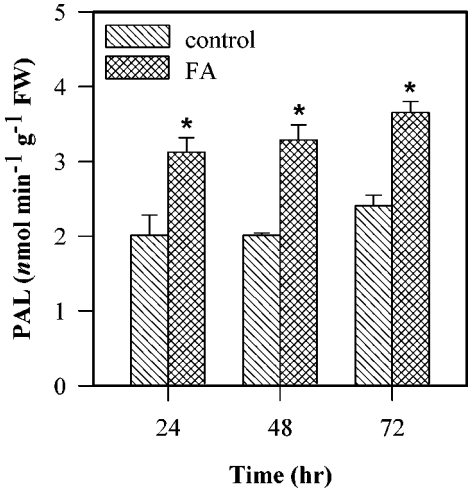

FIG. 2. Effects of FA on phenylalanine ammonia-lyase. *Values ($N = 4$ to 8 $\pm$ SEM) differing significantly from the control ($P < 0.05$).

of maize (*Zea mays* L.) seedlings affecting both shoots (>60%) and roots (>40%) (Devi and Prasad, 1996). At the same concentration, canola (*Brassica napus* L.), primary root length and root FW were reduced by about 35 and 43%, respectively (Ng et al., 2003). Application of 0.4 mM FA caused a considerable decrease in the

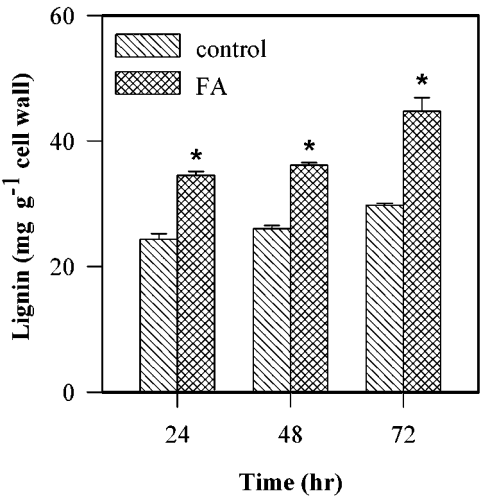

FIG. 3. Effects of FA on lignin contents. *Values ($N = 4$ to 8 $\pm$ SEM) differing significantly from the control ($P < 0.05$).

root length (>21%) and root DW (>30%) in sorghum (*Sorghum bicolor* Moench.) seedlings (Einhellig and Eckrich, 1984). At 0.25 mM, FA inhibited root elongation, stimulated secondary root initiation, and increased the root-shoot ratio in cucumber (*Cucumis sativus* L.) seedlings (Blum and Rebbeck, 1989). In soybean, Patterson (1981) showed that 1.0 mM FA significantly reduced total DW (>45%) in soybean (*Glycine max* L.).

FA increased POD activity in the present study. An increase of soluble POD activity is generally accompanied by a decrease in root growth. In cucumber (*Cucumis sativus* L.) and maize (*Zea mays* L.) roots treated with 1.0 mM FA, soluble POD increased significantly ($P < 0.05$) and was coupled with a drastic decrease in root length (Shann and Blum, 1987; Devi and Prasad, 1996; Politycka, 1996). These investigators attributed the effects of FA to production of free radicals. Soluble POD catalyzes the oxidation of structurally diverse phenolic substrates and is often regarded as an antioxidant enzyme that protects cells from the destructive influence of toxic oxygen radicals. However, if the capacity of the cells to scavenger oxygen radicals is exceeded, phenolic acid oxidation by soluble POD leads to production of quinones (Politycka, 1998). Consequently, these compounds may depolarize the root cell membrane and cause deterioration in membrane integrity by changing lipid composition (Ferrarese et al., 2001), inducing lipid peroxidation (Politycka, 1996; Doblinski et al., 2003), and affecting enzymatic activities (Devi and Prasad, 1996). Baziramakenga et al. (1995) reported that phenolic acids decreased sulfhydryl groups in soybean roots, suggesting that depletion may lead to inactivation of enzymes and disruption of the functions of carrier proteins. These intramolecular changes in protein sulfhydryl groups may result in enhanced permeability of the membranes causing electrolyte leakage and blocking plant nutrient uptake (Lyu and Blum, 1990).

Since POD may be bound to cell wall polymers (Whetten et al., 1998), an essential role for POD in the stiffening of cell walls through the formation of biphenyl bridges between wall polymers and, thus, reduction of the cell wall extensibility, has been proposed (Fry, 1986). It has also been suggested that FA may be esterified to cell wall polysaccharides or incorporated into lignin structures and cause cell wall rigidity and restriction of cell growth (Tan et al., 1992). FA dimerization in the hypocotyls of *Pinus pinaster* (A.), due to the oxidative capacity of the bound form of POD, was inversely related to its growth capacity (Sánchez et al., 1996). So, it is feasible that these facts may, in part, explain decreases in root growth reported in this paper (Table 1).

Increased activity of PAL, the primary enzyme of the phenylpropanoid biosynthetic pathway and lignin synthesis, may be a response of plants to various biotic and abiotic stresses. Unfortunately, few studies have been carried out on the effects of exogenous FA on PAL, and results are contradictory. Sato et al. (1982) pointed out that FA was ineffective on PAL of sweet potato (*Ipomea batatas* L.) and pea (*Pisum sativus* L.). FA was unable to affect PAL activity in cucumber (*Cucumis*

sativus L.) roots (Shann and Blum, 1987). In contrast, increased PAL activities is associated with a decrease in cucumber (*Cucumis sativus* L.) roots, after 24 and 48 hr of FA treatment (Politycka, 1998). The present data show that PAL activities increase in soybean after 24 to 72 hr of FA treatment (Figure 1). Lignin content also increased as a result of FA treatment (Figure 3). Shann and Blum (1987) showed that [^{14}C]-FA is incorporated into lignin residues isolated from cucumber (*Cucumis sativus* L.) roots. Concomitantly, they verified that an increase in lignin contents associated with a decrease in root growth. In maize (*Zea mays* L.) roots treated with FA, increased activity of cell wall-bound POD correlated with a significant increase in lignin content and reduction in root growth (Devi and Prasad, 1996). Politycka (1999) also verified that FA treatment of cucumber (*Cucumis sativus* L.) seedlings stimulated lignin production and was coupled with a decrease in root growth.

FA treatment of soybean seedlings results in increased cell wall-bound POD and PAL activity and lignin content and suggests that these enzymes probably participate in the regulation of root growth. FA-induced inhibition of roots of soybean seedlings may be in part due to cell wall stiffening resulting from the formation of cross-linking among cell wall polymers and lignin production. Induced lignification is, as a rule, accompanied by an increase in the activity of enzymes associated with the phenylpropanoid pathway, e.g., PAL, POD, and cinnamyl alcohol dehydrogenase (CAD), a highly specific lignification marker. This process appears to involve oxidative coupling, dependent on H_2O_2, which causes a rapid cross-linking of cell wall polymers (Bolwell and Wojtaszek, 1997; Wojtaszek, 1997). Thus, if cell wall-bound POD and PAL regulate cell wall stiffening and lignin polymerization, there must be a sufficient supply of H_2O_2. This relatively stable form of reactive oxygen species (ROS) may be produced by pH dependent cell wall peroxidases and by NADPH oxidase complex (Frahry and Schopfer, 1998; Chen and Schopfer, 1999; Ros-Barceló et al., 2002). The effect of FA on H_2O_2 levels and on CAD activity is needed to verify whatever FA-induced reduction in root growth results from increased lignification.

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IDENTIFICATION AND MOLECULAR CLONING OF PUTATIVE ODORANT-BINDING PROTEINS FROM THE AMERICAN PALM WEEVIL, *Rhynchophorus palmarum* L.

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Abstract—We have identified and cloned the cDNAs encoding two odorant-binding proteins (OBPs) from the American palm weevil (APW) *Rhynchophorus palmarum* (Coleoptera, Curculionidae). Degenerate primers were designed from the N-terminal sequences and were used in polymerase chain reaction (PCR) in order to obtain full-length sequences in both males and females. In both sexes, two different cDNAs were obtained, encoding 123 and 115 amino acid-deduced sequences. Each sequence showed few amino acid differences between the sexes. The proteins were named RpalOBP2 and RpalOBP4 for male, RpalOBP2' and RpalOBP4' for female, with the types 2 and 4 presenting only 34% identities. These proteins shared high identity with previously described coleopteran OBPs. In native gels, RpalOBP2 clearly separated into two bands and RpalOBP4 into three bands, suggesting the presence of several conformational isomers. Thus, OBP diversity in this species may rely on both the presence of OBPs from different classes and the occurrence of isoforms for each OBP.

Key Words—*Rhynchophorus palmarum*, Coleoptera, odorant-binding protein, molecular cloning, olfaction.

INTRODUCTION

The American palm weevil (APW), *Rhynchophorus palmarum* (Coleoptera, Curculionidae), is a severe pest of neotropical plantations of oil palm and coconut. The larvae cause direct damage by feeding in the entire plant, and the adults also are vectors of the nematode causing red ring disease (Griffith, 1987).

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Chemical communication in this species has been extensively studied with the goal of developing alternative control methods for this pest. The males are primarily attracted by semiochemicals emitted by host plants and a few key compounds are recognized by the insects among a complex pattern of odorants and are used to locate the host (Hernandez et al., 1992). The most attractive molecules are produced from the fermentation of tissues or sap (Nagnan et al., 1992; Rochat et al., 1993). Males also produce an aggregation pheromone, but only when they feed on the host plant (Rochat et al., 1991a). Pheromone release is also triggered by ethyl acetate (Jaffé et al., 1993), the major odor component of the suitable host plants. The pheromone was identified as (4*S*, 5*E*)-2-methylhept-5-en-4-ol and named rhynchophorol (Rochat et al., 1991b). It is secreted by prothoracic glands as a direct response to allelochemical stimuli (Sanchez et al., 1996).

Rhynchophorol acts as a long-range attractant and synergizes host kairomones to attract conspecific males and females (Oehlschlager et al., 1993; Rochat et al., 1993). Pheromone synergists have been identified (Rochat et al., 2000) and evoke electroantennogram responses similar to those elicited by the pheromone. In a recent study, Said et al. (2003) showed by single-cell recordings that a plant synergist, acetoin, stimulates the same neurons as the pheromone. Whereas the coding of the synergy has been attributed to the central nervous system (Carlsson and Hansson, 2002), these data suggest that a peripheral coding may also be involved. In insect antennae, odorant-binding proteins play an important role in the first coding of odorants as discriminating filters and transporters (Du and Prestwich, 1995; Wojtasek et al., 1999; Jacquin-Joly et al., 2000; Maida et al., 2000; Mohl et al., 2002). For example, in Coleoptera, different OBPs exhibit different affinities for the same ligand (Wojtasek et al., 1999), and OBP polymorphism related to the binding specificity is increasingly documented (see above). To lay down the molecular basis of the peripheral coding of the synergy, we investigated the diversity of the APW antennal proteins, by N-terminal microsequencing and molecular cloning of the encoding cDNAs in both sexes.

METHODS AND MATERIALS

Insects. Insects were caught during field experiments using traps containing either mixtures of synthetic allelochemicals or rhynchophorol (Oehlschlager et al., 1993; Rochat et al., 1993). They were sent within 2 d and only live insects were dissected.

Protein Extraction. Adult antennal clubs were crushed in Tris buffer (20 mM; pH = 7.4) with a Polytron™ homogenizer. This solution was filtered by centrifugation (15 min at 15,000 *g*) with Z-spin filters (Gelman Sciences) and the supernatant was evaporated under vacuum (Savant Speed-Vac). Other parts of the body were used as controls for the tissue-specificity of antennal proteins and prepared as described above. For each set of experiments, 40 males and 40 females were used.

Three independent sets of experiments were conducted with insects caught in the field at time intervals of 6 mo.

Electrophoresis and Protein Sequencing. Nondenaturing-polyacrylamide gel electrophoresis (native-PAGE, 16.8% acrylamide) was carried out at 150 V (constant voltage) and room temperature. For observation, the gels were stained overnight in a colloidal Coomassie blue R solution (12% trichloroacetic acid, 5% ethanolic solution of 0.035% Serva blue R 250) and rinsed in distilled water. For N-terminal determination, proteins were analyzed as above, then electroblotted onto ProBlott™ membranes (Applied Biosystems) for 1 hr at 400 mA (constant current) and 4°C. Membranes were stained with 0.2% Ponceau red in 1% acetic acid and destained in distilled water. The bands of interest were carefully cut, then processed for sequencing by J. D'Alayer (Institut Pasteur, France) with a gas-phase microsequencer (Applied Biosystems) using Edman degradation. In case of mixtures inside one band, each sequence was determined using quantitative sequencing data for each cycle. Sequence homologies were analyzed using Blastp algorithms (NCBI).

RNA Extraction and cDNA Synthesis. Total RNAs were extracted from 20 male and 20 female antennal clubs, respectively with Tri-Reagent™ (Euromedex). Single-stranded cDNA was synthesized from approximately 1 μg of total RNA with M-MLV (Clontech), using the Advantage RT for PCR kit (Clontech). The reaction mixture contained dNTP mix, Rnasin, sterile water, and template RNA to a final volume of 20 μl. The mix was heated at 68°C for 10 min and chilled on ice before adding the M-MLV (600 u), then incubated 1 hr at 42°C and finally the reverse transcriptase was inactivated at 95°C for 5 min.

3′ RACE-Polymerase Chain Reaction and Molecular Cloning. Approximately 1 ng of cDNA from both male and female *R. palmarum* clubs was used for polymerase chain reaction. Degenerate sense primers were designed on the basis of the protein KFDDSI and DEMKELA N-terminal sequences determined by microsequencing: *RpalOBP2sens* AAGTTCGAYGAYWSIAT and *RpalOBP4sens* GAYGARATG AARGARYTI, and were synthesized by Isoprim. These primers were used in a pair with an oligodT_{18}-anchor CATGCATGCGGCCGCAAGCT_{18}VN (Isoprim) in order to perform the 3′ RACE. Polymerase chain reactions were carried out with Taq polymerase (1 u, Promega) in 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP. Forty amplification cycles were performed with annealing temperatures of 40 and 45°C for *RpalOBP2sens* and *RpalOBP4sens*, respectively. The amplified cDNAs were purified after agarose electrophoresis using GenElute (Supelco) and ligated into the plasmid pCR™-II using the TOPO cloning kit (Invitrogen). After transformation, positive clones were digested with EcoRI (Biolabs) to screen for the presence of insert. Recombinant plasmids were then isolated using Plasmid Midi kit (Qiagen), and both strands were subjected to automated sequencing with vector primers (T7, M13 promoter) by ESGS (Evry, France) and Genomexpress. Data base searches

were performed with the Blastn program (NCBI) and sequence alignment with the Clustalw (NPS@ NPSA, Combet et al., 2000).

RESULTS

Protein Electrophoresis and N-Terminal Microsequencing. Crude extracts of antennal clubs were first analyzed by electrophoresis in native conditions. Coomassie blue staining revealed the presence of four major bands, which migrated to the same position for both male and female extracts (Figure 1) and were absent in other tissues (data not shown). Aliquots of the same extracts were used for protein sequencing: after native-PAGE, the proteins were transferred onto ProBlott membranes, and the bands were cut out and analyzed for N-terminal determination. Four different N-terminal sequences were found in male and female extracts: XLSDEQKQKV (RpalOBP1), KFDDSIISDD (RpalOBP2), AMTSEQRERF (RpalOBP3), and ISDEMKELAQ (RpalOBP4). In male extracts, bands II and III each contained a mixture of RpalOBP2, RpalOBP3, and RpalOBP4 sequences, and band IV contained the RpalOBP4 sequence alone. In female extracts, the same distribution was observed, except that RpalOBP4 was not detectable in band II. The relative percentages of each sequence in the four bands for male and female extracts are indicated in Table 1. The difference in percentages is high enough to allow assignment of a residue to a sequence with good confidence. This polymorphism did not result from contamination during the cutting of bands, because they were well separated in native-PAGE and cut precisely.

The two most abundant sequences, RpalOBP2 and RpalOBP4, found in three different protein bands, were used to design degenerate primers to amplify the encoding cDNAs by PCR, and to obtain the full-length sequences.

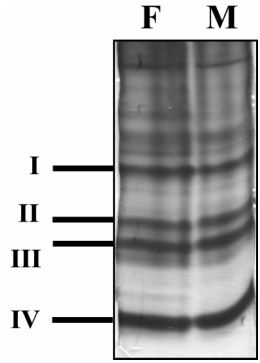

FIG. 1. Soluble antennal proteins from female (F) and male (M) *R. palmarum* separated on 16.8% native-PAGE. Coomassie blue staining. Bands are numbered according to increasing acidity (I → IV).

TABLE 1. N-TERMINAL SEQUENCES IDENTIFIED IN MALE AND FEMALE PROTEIN BANDS (NUMBERED IN FIGURE 1)

Band #	Male	Female
I	XLSDEQKQKV (100)	XLSDEQKQKV (100)
	KFDDSIISDD (73)	KFDDSIISDD (90)
II	AMTSEQRERF (21)	AMTSEQRERF (10)
	ISDEMKELAQ (6)	
	KFDDSIISDD (87)	KFDDSIISDD (78)
III	AMTSEQRERF (6)	AMTSEQRERF (14)
	ISDEMKELAQ (7)	ISDEMKELAQ (8)
IV	ISDEMKELAQ (100)	ISDEMKELAQ (100)

Note. The relative percentage of each sequence in the band, calculated from the initial yield of sequencing, is indicated in brackets.

Cloning of OBP-Type Sequences. PCR reactions with the designed primers resulted in amplification of cDNAs, designated *RpalOBP2* and *RpalOBP4* in males (Figure 2) and *RpalOBP2′* and *RpalOBP4′* in females. The sequences were deposited in the GenBank database under the accession numbers AF139912 (*RpalOBP2* cDNA,) AF141865 (*RpalOBP2′* cDNA), AY182011 (*RpalOBP4* cDNA) and AY394703 (*RpalOBP4′* cDNA).

The male *RpalOBP2* and female *RpalOBP2′* cDNAs encode proteins of 123 amino acids (Figure 2) with calculated molecular masses of 13,831 Da and 13,805 Da, and isoelectric points of 5.3 and 5.19 for males and females, respectively (MW-CALC, Infobiogen). The RpalOBP2 and RpalOBP2′ proteins only differ by three substitutions, of which two are homologues ($I_{56} \rightarrow V$, $I_{98} \rightarrow V$), and one substitutes an asparagine (N_{100}) for aspartic acid, which slightly modifes the isoelectric point, without consequence to the comigration of the two proteins in native gels (Figure 1). RpalOBP4 (male) and RpalOBP4′ (female) protein sequences consist of 115 amino acids with only one substitution (E_{84}ПК) (Figure 2), with calculated molecular masses of 13,069 and 13,068 Da, and isoelectric points of 4.40 and 4.51 for males and females, respectively. Because the sense primers were designed corresponding to the N-terminal sequences of the proteins, cloned cDNAs encoded only the sequence of mature proteins without signal peptide sequences. Female RpalOBP2′ and RpalOBP4′ share 97.5% and 99% identities with male RpalOBP2 and RpalOBP4, respectively, whereas RpalOBP2 and RpalOBP4 share only 34.15% amino acid identities.

The Blast search analysis on RpalOBP2 and RpalOBP4 indicated significant identities to previously described OBPs from other insects (Table 2). RpalOBP4 and 4′ are approximately 50% identical to various OBPs and PBPs from coleopteran species (Figure 3). RpalOBP2 and 2′, however, are less similar than RpalOBP4 to other coleopteran OBPs, with an average of 30%. Although the observed identities

RpalOBP2

```
 K   F   D   D   S   I   I   S   D   D   I   K   K   L   L   K   G   L      18
AAG TTC GAT GAT TCG ATA ATA TCA GAT GAT ATT AAA AAA CTT CTC AAA GGT TTA     54

 H   D   V   C   V   G   K   I   G   V   E   E   A   L   I   E   N   L      36
CAT GAT GTT TGT GTC GGC AAA ATA GGC GTT GAA GAA GCA TTA ATT GAA AAT CTA    108

 K   N   A   E   F   T   E   D   D   K   L   K   C   Y   V   H   C   L      54
AAA AAT GCC GAA TTT ACT GAA GAT GAT AAA TTA AAA TGT TAT GTA CAT TGT CTT    162

 L   I   Q   V   G   A   M   D   L   A   G   H   I   D   A   E   A   A      72
CTT ATC CAA GTA GGC GCT ATG GAC CTG GCA GGA CAT ATA GAC GCA GAG GCA GCC    216

 I   E   L   I   P   E   Q   I   R   V   S   V   I   Q   E   A   N   K      90
ATA GAA CTG ATT CCA GAA CAA ATC AGA GTC TCT GTG ATT CAG GAG GCC AAC AAG    270

 C   A   K   D   K   E   K   I   E   N   H   C   S   R   A   F   A   T     108
TGT GCT AAA GAT AAA GAA AAA ATT GAA AAT CAC TGT AGT CGA GCC TTT GCT ACG    324

 I   K   C   L   H   D   V   N   P   D   I   Y   Y   M   F   *             123
ATA AAA TGT CTT CAC GAC GTT AAT CCC GAT ATC TAC TAT ATG TTT  tgaaaaaaaat   380

cttggtaaactaaaataattgtagataaatagtcatgtagtgataattacttaaatatattacaatattatg   452

caaccaaaaaaaaaaaaaaaaaaaa                                                  479
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RpalOBP4

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 I   S   D   E   M   K   E   L   A   Q   Q   L   H   N   T   C   V   S      18
         GAC GAG ATG AAG GAA TTG GCT CAG CAG TTA CAT AAC ACA TGT GTG TCT     54

 E   T   G   T   T   E   D   A   I   T   N   A   R   A   G   T   F   T      36
GAA ACA GGC ACA ACT GAG GAT GCA ATT ACC AAT GCT AGA GCT GGT ACA TTT ACG    108

 D   D   E   K   F   K   C   Y   L   K   C   L   L   D   Q   M   A   I      54
GAC GAT GAG AAA TTT AAA TGT TAT TTG AAA TGT TTG CTT GAC CAA ATG GCT ATA    162

 V   D   E   E   G   R   I   D   V   E   A   M   I   A   V   L   P   E      72
GTA GAT GAA GAA GGC AGA ATT GAT GTC GAA GCA ATG ATT GCA GTA TTA CCA GAA    216

 E   F   Q   D   S   L   P   P   V   I   R   E   C   D   T   I   I   G      90
GAA TTT CAA GAT AGT TTG CCA CCA GTT ATT AGA GAA TGT GAC ACA ATA ATA GGA    270

 A   N   A   C   D   N   V   W   L   T   Q   Q   C   Y   Y   K   E   N     108
GCA AAT GCT TGT GAT AAC GTT TGG CTT ACC CAG CAA TGT TAT TAC AAA GAA AAC    324

 P   E   H   Y   F   L   I   *                                             115
CCC GAA CAT TAT TTC TTA ATT  taattgagctaaatcctgccgcactgacacggataaccgaaat   388

aactgtatatataacaatatactctctaagtttgtttttctgagatctgtttgaatatatgcgtactaatgc   460

aatgaaaaaaaaaaaaaaaaa                                                      482
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FIG. 2. Nucleotide and deduced amino acid sequences of male RpalOBP2 and RpalOBP4. (GenBank accession no. AF139912 and AY182011, respectively). The N-terminal sequences obtained by microsequencing are underlined. The stop codons at the termination site are indicated by an asterisk.

TABLE 2. OBP SEQUENCES FROM COLEOPTERA USED IN CLUSTAL W

	Species	Protein names	GenBank accession numbers	References
Curcujiformia	*Rhyncophorus palmarum*	RpalOBP2	AF139912	This publication
		RpalOBP2′	AF141865	This publication
		RpalOBP4	AY182011	This publication
		RpalOBP4′	AY394703	This publication
Scarabaeiformia	*Anomala osakana*	AosaPBP	AF031492	Wojtasek et al., 1998
	Anomala octiescostata	AoctPBP	AB040143	Nikonov et al., 2002
	Anomala cuprea	AcupPBP	AB040141	Nikonov et al., 2002
	Exomala orientalis	EoriPBP	AB040144	Peng and Leal, 2001
	Heptophylla picea	HpicOBP1	AB025571	Deyu and Leal, 2002
	Holotrichia parallela	HparOBP1	AB026555	Deyu and Leal, 2002
	Phyllopertha diversa	PdivOBP1	AB026552	Wojtasek et al., 1999
		PdivOBP2	AB026553	Wojtasek et al., 1999
	Popillia japonica	PjapPBP	AF031491	Wojtasek et al., 1998

```
                                10        20        30        40        50        60        70
                                |         |         |         |         |         |         |
R. palmarum OBP2        ------KFDDSIISDDIKKLLKGLHDVCVGKIGVEEALIENLKNAE-FTEDDKLKCYVHCLLIQVGAMDLA
R. palmarum OBP2'       ------KFDDSIISDDIKKLLKGLHDVCVGKIGVEEALIENLKNAE-FTEDDKLKCYVHCLLVQVGAMDLA
R. palmarum OBP4        --------------DEMKELAQQLHNTCVSETGTTEDAITNARAGT-FTDDEKFKCYLKCLLDQMAIVDEE
R. palmarum OBP4'       --------------DEMKELAQQLHNTCVSETGTTEDAITNARAGT-FTDDEKFKCYLKCLLDQMAIVDEE
P. diversa OBP1         --------------EEMEELAKQLHNDCVGQTGVDEAHITTVKDQKGFPDDEKFKCYLKCLMTEMAIVGDD
P. diversa OBP2         ----------KEHGQKVLEQIIDYATSCADSLGVSPEDMKLLMEKK-FPTSREGQCMPSCVNKKFGLQKAD
A. osakana PBP          ------------MSEEMEELAKQLHNDCVAQTGVDEAHITTVKDQKGFPDDEKFKCYLKCLMTEMAIVGDD
A. octiescostata PBP    ------------MSEEMEELAKQLHNDCVAQTGVDEAHITTVKDQKGFPDDEKFKCYLKCLMTEMAIVGDD
E. orientalis PBP       ------------MSEEMEELAKQLHDDCVGQTGVDEAHITTVKDQKGFPDDEKFKCYLKCLMTEMAIVGDD
P. japonica PBP         ------------MSEEMEELAKQLHDDCVSQTGVDEAHITTVKDQKGFPDDEKFKCYLKCLMTEMAIVGDD
A. cuprea PBP1          ------------MSEEMEELAKQLHNDCVGQTGVDEAHITTVKDQKGFPDDEKFKCYLKCLMTEMAIVGDD
H. picea OBP1           --------------EEMEELAKQLHDDCVGQTGVDEAHIGTVKDQKGFPDDEKFKCYLKCLMTEMAIVGDD
H. parallela OBP1       --------------EEMEELAKQLHDDCVGQTGVDEAHIGTVKDQKGFPDDEKFKCYLKCLMTEMAIVGDD

                                80        90        100       110       120       130       140
                                |         |         |         |         |         |         |
R. palmarum OBP2        GHIDAE------AAIELIPEQIRVSVIQEANKCAKDKEKIENHCSRAFATIKCLHDVNPDIYYMF-----
R. palmarum OBP2'       GHIDAE------AAIELIPEQIRVSVIQEANKCAKDKEKVEDHCSRAFATIKCLHDVNPDIYYMF-----
R. palmarum OBP4        GRIDVE------AMIAVLPEEFQDSLPPVIRECDTIIGANACDNVWLTQQCYYKENPEHYFLI-----
R. palmarum OBP4'       GRIDVE------AMIAVLPEEFQDSLPPVIRKCDTIIGANACDNVWLTQQCYYKENPEHYFLI-----
P. diversa OBP1         GIVDIE------AAVGVLPDELKAKAEPVMRKCGFKPGANPCDNVYQTHKCYFETDPQSYMIV-----
P. diversa OBP2         GTLNKEYRYSEMENVKAIDEEIYNKMNSVWDKCVINGADGTDECDTGMKVVTCMKEESEKLGLSKDAIGF
A. osakana PBP          GVVDIE------AAVGVMPDEYKAKAEPVIRKCGVIPGANPCDNVYQTHKCYYDTDPQSYMIV-----
A. octiescostata PBP    GVVDVE------AAVGVLPDEYKAKAEPVMRKCGVKPGANPCDNVYQTHKCYYETDPQSYMIV-----
E. orientalis PBP       GIVDVE------AAVGVIPDEYKAKAEPIMRKCGFKPGANPCDNVYQTHKCYYDTDPQAYMII-----
P. japonica PBP         GVVDVE------AAVGVLPDELKAKAEPIMRKCGFKPGANPCDNVYQTHKCYYETDAQSYMIV-----
A. cuprea PBP1          GIVDIE------AAVGVLPDELKAKAEPVMRKCGFKPGANPCDNVYQTHKCYFETDPHSYMIV-----
H. picea OBP1           GVVDVE------AAVGVLPDEFKDKAEPIMRKCGVKPGANPCDNVYQTHKCYYDADPNSYMIV-----
H. parallela OBP1       GVVDVE------AAVGVLPDEFKDKAEPIMRKCGVKPGANPCDNVYQTHKCYYDADPNSYMIV-----
```

FIG. 3. Multiple sequence alignment between *R. palmarum* sequences and other coleopteran OBPs. Sequences used in this alignment are listed in Table 2. The six conserved cysteines are in bold.

between all those proteins are moderate, six cysteines are conserved. The high degree of conservation suggests that the cysteines play an important role in these proteins (Leal et al., 1999; Scaloni et al., 1999). Moreover, if the comparison between these proteins is based upon homologies, the percentages reach values between 55 and 76, the highest being obtained with beetle PBPs.

DISCUSSION

The protein content of antennal clubs from male and female APW has been analyzed and showed heterogeneity of odorant-binding proteins. Four OBPs were characterized in both sexes by microsequencing of the N-terminus of these proteins. Oligonucleotide probes were deduced from the N-terminal of the two most abundant proteins that led to the cloning and sequencing of two cDNAs encoding RpalOBP2 and RpalOBP4 OBPs in male, with similar orthologous cDNAs in female, RpalOBP2′ and RpalOBP4′. The presence of several antennal specific proteins in *R. palmarum* brings a new example of OBP diversity in beetles. Whereas in some species, such as *Popillia japonica* and *Anomala osakana* (Table 2), only one OBP could be detected (Wojtasek et al., 1998), two proteins were also characterized in *Phyllopertha diversa* (Wojtasek et al., 1999). The detection of OBPs in both sexes is consistent with other observations in beetles, for example in *Anomala cuprea* and *A. octiescotata* (Nikonov et al., 2002). Although the two PBPs described in these species are identical in both sexes, here the cDNA sequences obtained in male and female *R. palmarum* are slightly different, suggesting population polymorphism rather than possible allelic variation. Indeed, cDNAs were prepared from a pool of individuals and nucleotide differences often occur at the third codon position and do not change the amino acid coding. A previous example of OBP alleles encoded at the same locus has been reported in the Lepidoptera *Epiphyas postvittana*, (Newcomb et al., 2002). In addition, there is no support for OBP sexual dimorphism from any behavioral, morphological, or electrophysiological data from *R. palmarum*. In both sexes, the olfactory sensilla are restricted to the distal part of the antenna, the antennal club, and distributed without localization of functional types (Said et al., 2003). Males and females behave equally in response to the detection of host-plant odorants or the male produced aggregation pheromone (Said, 2003). In addition, single-cell recordings did not show any sexual dimorphism whatever the compound tested (Said et al., 2003). More experiments are needed to determine if the OBP sequence variability in *R. palmarum* comes from allelic variation or gene duplication (Merritt et al., 1998).

The four protein sequences were found in several bands in native-PAGE, indicating different shape and global charge. This can be explained by either the presence of genetic variants (allelic products of the same gene) or by conformational isomers of the same primary sequence. This source of diversity has been proposed

for the lepidopteran MbraPBP1 from *Mamestra brassicae* (Nagnan-Le Meillour et al., 1996; Nagnan-Le Meillour and Jacquin-Joly, 2003) and with supporting evidence from scarab beetle OBPs from *Holotrichia parallela* and *Heptophylla picea* (Deyu and Leal, 2002). Moreover, different isoforms of *P. diversa* OBP have been shown to have different binding affinities for diverse pheromone components (Wojtasek et al., 1999).

Here, the diversity of OBPs in *R. palmarum* could come from the expression of several different genes, at least RpalOBP1 to RpalOBP4, together with the formation of conformational isomers for each primary sequence (up to 3 for RpalOBP4, present in distinct bands II, III, and IV). The diversity of OBPs is a crucial question affecting their binding properties to odorant ligands. Despite the lack of binding data in *R. palmarum*, strong homologies between RpalOBP sequences and various beetle proteins, in particular PjapPBP and AosaPBP that bind pheromones *in vitro* (Wojtasek et al., 1998), suggest that APW male and female OBPs are related to the PBP class. Moreover, as the different sequences show poor homologies between each other (34.1%), they could belong to different classes of OBPs, tuned to the binding of structurally different ligands. The APW semiochemicals are mainly small volatile molecules (rhynchophorol: (4*S*, 5*E*)-2-methylhept-5-en-4-ol) structurally closer to plant volatiles than to pheromones of Lepidoptera. Binding assays with tritiated ligands are in progress to determine the specific ligands of the APW OBPs described here and the putative involvement of these two different OBPs in the pheromone-plant odorants synergy mechanism.

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CHIRAL SYNTHESIS OF (*Z*)-3-*cis*-6,7-*cis*-9, 10-DIEPOXYHENICOSENES, SEX PHEROMONE COMPONENTS OF THE SATIN MOTH, *Leucoma salicis*

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Abstract—All four isomers of (*Z*)-3-*cis*-6,7-*cis*-9, 10-diepoxyhenicosenes, **1**–**4**, have been synthesized using D-xylose as the chirally pure starting material. D-Xylose was first converted to 2-deoxy-4,5-*O*-isopropylidene-3-*t*-butyldimethylsilyl-D-*threo*pentose **11**, via several steps of selective protection, dehydroxylation, and deprotection. Wittig coupling of **11** with nonyltriphenylphosphonium bromide followed by hydrogenation and acid catalyzed deprotection of hydroxyl groups yielded the chiral (2*R*,3*R*)-1,2,3-triol, **14**, which was used as the precursor for the C-8 to C-21 unit of the (*Z*)-3-*cis*-6,7-*cis*-9,10-diepoxyhenicosenes. Selective tosylation of **14** followed by stereospecific cyclization yielded (2*R*,3*R*)-1,2-epoxytetradecan-3-ol, **16**, which was then divergently converted to the *t*-butyldimethylsilyl ether **17** and tosylate **22**, respectively. Establishment of the C-5 through C-7 unit of the target molecules was accomplished via regiospecific coupling of **17** with 1-*t*-butyldimethylsiloxy-2-propyne to form **18**. Stepwise transformation of **18** via the formation of tosylate **19**, desilylation, and stereospecific cyclization to form epoxy alcohol **20**, followed by P2–Ni reduction yielded a key intermediate, allylic epoxy alcohol (*Z*)-2-(5*S*, 6*R*)-*cis*-5,6-epoxyheptadecen-1-ol, **21**. Similarly, the coupling of **22** with 1-*t*-butyldimethylsiloxy-2-propyne yielded **23**, which was stereospecifically cyclized to form **24**. Desilylation and P2–Ni reduction of **24** gave the antipodal intermediate, (*Z*)-2-(5*R*, 6*S*)-*cis*-5,6-epoxyheptadecen-1-ol, **26**. Asymmetric epoxidation of antipodes **21** and **26** with (L)- or (D)-diethyl tartrates resulted in the formation of diepoxy alcohols **27** and **29** from **21**, and **33** and **31** from **26**, respectively. Tosylation of these diepoxy alcohols followed by coupling with lithium dibutenyl cuprate yielded the four stereoisomers of (*Z*)-3-*cis*-6,7-*cis*-9, 10-diepoxyhenicosenes, **1**–**4**. Analysis of the retention characteristics of these

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materials revealed that one or both of the S*,R*,S*,R* stereoisomers comprise the major pheromone component(s) of *Leucoma salicis*.

Key Words—Chiral synthesis, moth sex pheromone, diepoxyhenicosene, Satin moth, *Leucoma salicis*, leucomalure, D-xylose, Katsuki-Sharpless oxidation.

INTRODUCTION

Epoxides with different chain lengths and epoxy positions have been identified as lepidopteran sex pheromones, principally among the geometrid, lymantrid, and noctuid moths (Mayer and McLaughlin, 1991; Arn, 2000; Ando, 2003). Structurally similar epoxy pheromones are found in different species of moths, and reproductive isolation is often, but not always, maintained by stereoisomer chirality (Mayer and McLaughlin, 1991; Arn, 2000; Ando, 2003; Millar et al., 1990; Millar, 2000). In some species, the presence of a trace amount of the wrong antipode can strongly inhibit biological activity (Plimmer et al., 1977; Cardé et al., 1978; Gries et al., 1996). (*Z*)-3-*cis*-6,7-*cis*-9,10-Diepoxyhenicosenes have been identified as principal sex pheromone components of the Satin moth, *Leucoma salicis* L., an important defoliator of deciduous trees in Europe, Western Asia, and North America (Gries et al., 1997). The related compounds (*Z*)-9-*cis*-3,4-*cis*-6,7-diepoxyhenicosene also has been identified as components of the sex pheromone of the clear-winged tussock moth, *Perina nuda* (Yamazawa et al., 2001; Wakamura et al., 2002).

A synthetic racemic mixture of one of the diastereoisomers of (*Z*)-3-*cis*-6,7-*cis*-9,10-diepoxyhenicosenes was strongly attractive to male satin moths in field trapping experiments (Gries et al., 1997). However, the diastereomeric and chiral identity of the natural active component and the effect of the other stereoisomers were not determined. Synthesis and identification of the biologically active stereoisomer(s) is necessary to fully establish the identity of the pheromone and important to develop appropriate monitoring and control methods for satin moth populations.

Chiral diepoxyhenicosenes have been prepared by the partial epoxidation of monoepoxyhenicosadienes, followed by chiral HPLC separation of the resulting diastereomeric mixtures (Yamamoto et al., 1999). This methodology has provided all stereoisomers of the diepoxyoctadecenes,-nonadecenes, and -henicosenes derived from their (*Z*,*Z*,*Z*)-3,6,9-triene parents (Yamazawa et al., 2001). A second methodology involved the formation of racemic (*Z*)-3-*cis*-6,7-*cis*-9,10-diepoxyhenicosene starting with (*Z*)-2-butene-1,4-diol (Razkin Lizarraga and Mori, 2001). All four chiral isomers were similarly fashioned from a chiral synthon produced by resolution of racemic 4-(*t*-butyldiphenylsilyloxy)-*cis*-2,3-epoxy-1-butanol using Amano lipase PS-C (Muto and Mori, 2003). We report here the chiral synthesis of all four isomers of (*Z*)-3-*cis*-6,7-*cis*-9,10-diepoxyhenicosenes, **1**–**4** (Figure 1) from chirally pure D-xylose.

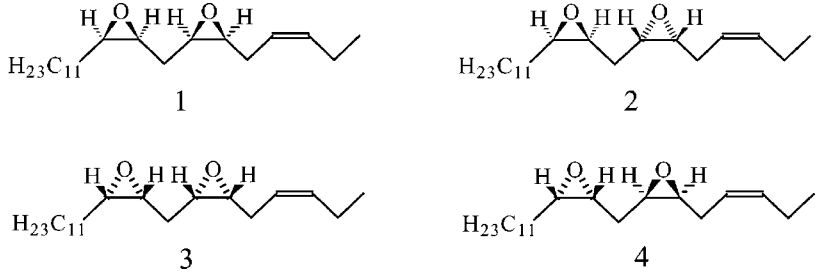


FIG. 1. The four (*Z*)-3-*cis*-6,7-*cis*-9,10-diepoxyhenicosenes.

METHODS AND MATERIALS

Solvents and reagents were used as received unless otherwise indicated. Solvent extracts of reaction products were routinely washed with dilute HCl, 10% $NaHCO_3$, water, and brine, and then dried over anhydrous Na_2SO_4, and concentrated under reduced pressure below 40°C unless otherwise stated. Gas chromatography was performed with an Hewlett-Packard (H-P) 5880 GC equipped with a DB-5 column (30 m × 0.25 mm i.d., J&W Scientific, Folsom, CA). Thin layer chromatography was carried out using silica gel 60G coated aluminum plates (Merck, DC-Alufolien Kieselgel 60 F_{254}). For visualization of compounds, plates were sprayed with 10% aq. H_2SO_4 and heated. Medium-pressure column chromatography was carried out using 230–400 mesh silica gel 60 (Mallinckrodt). HPLC was performed with a Waters LC625 instrument with a Waters 486 variable wavelength UV-visible detector. Nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) spectra were measured in $CDCl_3$ or C_6D_6 at 400 MHz (*J* values in Hz) on a Bruker AMX-400 spectrometer. Mass spectra were obtained on an H-P 5985B mass spectrometer equipped with a DB-5 column (30 m × 0.25 mm i.d.) with isobutane as reagent gas in chemical ionization mode. Infrared (IR) spectra were obtained as neat films on NaCl plates or as a KBr disc, on a Perkin-Elmer 1600 FTIR instrument. Melting points were determined on a Fisher–Johns apparatus and are uncorrected. Optical rotations were measured on an Autopol II automatic polarimeter. Concentrations for optical rotations are reported in grams/100 ml of solvent. Elemental analyses were done on a Carlo Erba model 1106 elemental analyzer. THF was distilled from Na/benzophenone, and HMPA and CH_2Cl_2 were distilled from CaH_2. Anhydrous *t*-butyl hydroperoxide was prepared by the procedure described by Hill et al. (1983). CuI was purified by extraction with dry THF in a Soxhlet extractor for 36 hr, and dried under vacuum.

D-*Xylose Diethyl Dithioacetal* ***6***. D-Xylose (Sigma, St. Louis, MO) (30 g, 0.2 mol) was dissolved in conc. HCl (25 ml) and stirred at 0°C in an ice bath. Ethanethiol (35 ml, 0.4 mol) was added to the mixture in small portions over

10 min. The ice bath was removed and the mixture stirred at room temperature for 30 min, cooled again to 0°C, and neutralized with NH_4OH solution (30 ml, 17 M). The mixture was extracted with hexane (4 × 100 ml) to remove unreacted ethanethiol. The aqueous layer was concentrated under vacuum to give an impure solid, which was dissolved in acetone/ethyl acetate (700 ml, 2:1), filtered through a Celite pad and concentrated. The residue was recrystallized from CH_2Cl_2/ether to yield **6** (51 g, 99%); $[a]_D^{24}$ −30.6° (c 0.98, H_2O), m.p.: 64–65°C [lit. Wolfrom et al., 1931; $[a]_D^{24}$ −31.2° (c 0.40, H_2O), m.p.: 63–65°C)].

*2,3:4,5-Di-O-Isopropylidene-*D*-xylose Diethyl Dithioacetal **7**.* A mixture of **6** (50 g, 0.19 mol) and iodine (8 g, 0.03 mol) in acetone (1500 ml) was stirred at room temperature overnight. The mixture was then poured into saturated $Na_2S_2O_3$ (500 ml) and stirred for 15 min. The acetone layer was separated and the aqueous layer was extracted five times with ether/hexane. The combined organic extracts were washed with saturated $Na_2S_2O_3$ (300 ml), water, and saturated NaCl and dried. The organic layer was filtered through a silica gel bed and concentrated to yield **7** as a syrup, 98% pure by GC (58 g, 88.4%). This product was used directly for the next step. $[a]_D^{24}$-54.9° (c 2.0, $CHCl_3$) [lit. Rollin and Pougny, 1986; $[a]_D^{20}$ −65° (c 1.91, acetone)].

*2-Deoxy-4,5-O-isopropylidene-*D*-threo-pent-1-enose Diethyl Dithioacetal **8**.* Compound **8** was prepared from **7** (56 g) by the procedure of Wong and Gray (1978), and purified by chromatography on silica gel (42 g, 91%); $[a]_D^{24}$ −43.6° (c 2.0, $CHCl_3$) [lit. Wong and Gray, 1978; $[a]_D^{23}$ −48.5° (c 2.4, $CHCl_3$)].

*2-Deoxy-4,5-O-isopropylidene-*D*-threo-pentose Diethyl Dithioacetal **9**.* This compound was prepared according to method A of Rollin and Pougny (1986). The crude product was chromatographed on silica gel (ether:hexane—3:1) to yield **9** (34 g, 86%); $[a]_D^{24}$ +22.6° (c 2.5, $CHCl_3$) [lit. Rollin and Pougny, 1986; $[a]_D^{23}$ +25.6° (c 5.0, $CHCl_3$)].

*2-Deoxy-4,5-O-isopropylidene-3-t-butyldimethylsilyl-*D*-threo-pentose diethyl dithioacetal **10**.* Compound **9** (33 g, 0.11 mol) was added to a mixture of TBDMSCl (21.5 g, 0.14 mol), Et_3N (100 ml), and DMAP (0.5 g) in dry DMF (100 ml) at room temperature, and the mixture was stirred overnight. Water (300 mL) was added, and the mixture was extracted with ether/hexane (5 × 200 ml). The organic layer was washed as usual, dried, and concentrated, and the residue was chromatographed on silica gel (ether:hexane—1:1) to yield **10** (41 g, 94%); $[a]_D^{24}$ +14.6° (c 1.0, $CHCl_3$). IR (film): 2930, 2857, 1460, 1373, 1254, 1212, 1157, 932, 835, 777 cm^{-1}. ^1H NMR ($CDCl_3$) ppm: 0.12 (s, 6H, —Si($C\underline{H}_3$)$_2$), 0.90 (s, 9H, —Si(C($C\underline{H}_3$)$_3$)), 1.24 (t, 3H, J = 7.4 Hz, —SCH_2-$C\underline{H}_3$), 1.25 (t, 3H, J = 7.4 Hz, —$SCH_2C\underline{H}_3$), 1.32 (s, 3H, —O—C($C\underline{H}_3$) 2—O—), 1.41 (s, 3H, —O—C($C\underline{H}_3$)$_2$—O—), 1.80 (ddd, 1H, J = 14.2, 10.2, 3.1 Hz, H′-2), 1.86 (ddd, 1H, J = 14.2, 8.3, 4.5 Hz, H″-2), 2.52–2.73 (m, 4H, —($SCH_2C\underline{H}_3$)$_2$), 3.75 (dd, 1H, J = 8.4, 6.5 Hz, H′-5), 3.95 (dd, 1H, J = 8.4, 6.6 Hz, H″-5), 4.00 (dd, 1H, J = 10.2, 4.5 Hz, H-1), 4.05–4.15 (m, 2H, H-3 and H-4). ^{13}C NMR ($CDCl_3$) ppm:

109.32, 78.22, 70.74, 65.38, 47.64, 38.83, 26.39, 25.93, 25.05, 24.42, 23.08, 18.15, 14.53, 14.43, −4.16, −4.58. CIMS: 396 (M^+, 7), 333 (68), 276 (28), 275 (100), 143 (45). Anal. calcd. for $C_{18}H_{38}O_3S_2Si$: C, 54.79; H, 9.72; found C, 54.60; H, 9.67.

2-Deoxy-4,5-O-isopropylidene-3-t-butyldimethylsilyl-D-*threo-pentose* ***11***. Mercuric oxide (43.3 g, 0.2 mol) and boron trifluoride–etherate (BF_3-Et_2O) (24.6 ml, 0.2 mol) in 85% aqueous THF (300 ml) were stirred vigorously under Ar atmosphere at room temperature. A solution of **10** (39 g, 0.1 mol) in THF (75 mL) was added to the solution by cannula over 10 min, and stirring was continued for 20 min. The mixture was filtered, and the residue was washed with ether (2 × 100 ml). The combined organic layers were poured into H_2O (300 ml) and extracted with ether/hexane (5 × 200 ml). The organic extracts were washed as usual, dried, filtered through a silica gel bed, and concentrated. The crude product was chromatographed on silica gel column (ether:hexane—1:1) to yield **11** (24.6 g, 86%); $[a]_D^{24}$ +24.1° (c 1.0, $CHCl_3$). IR (film): 2858, 2720, 1727, 1468, 1377, 1255, 1212, 1107 cm^{-1}. ^1H NMR ($CDCl_3$): 0.12 (s, 6H, $Si(CH_3)_2$), 0.90 (s, 9H, —$Si(C(C\underline{H}_3)_3)$), 1.34 (s, 3H, —O—$C(C\underline{H}_3)_2$—O—), 1.43 (s, 3H, —O—$C(C\underline{H}_3)_2$—O—), 2.50 (ddd, 1H, J = 16.2, 7.3, 2.7 Hz, H′-2), 2.63 (ddd, 1H, J = 16.2, 4.7, 1.6 Hz, H″-2), 3.83 (dd, 1H, J = 8.6, 6.3 Hz, H′-5), 3.97 (dd, 1H, 8.6, 6.9 Hz, H″-5), 4.15 (ddd, 1H, J = 6.9, 6.3, 5.1 Hz, H-4), 4.36 (ddd, 1H, J = 7.3, 5.1, 4.7 Hz, H-3), 9.80 (m, 1H, H-1). ^{13}C NMR ($CDCl_3$): 200.58, 109.67, 77.61, 68.27, 65.00, 46.64, 26.19, 25.68, 24.85, 17.96, −4.60, −4.87. CIMS: 289 (M^++1, 7), 271 (7), 245 (3), 231 (100), 187 (7), 173 (15), 145 (11), 139 (11), 115 (3), 101 (7). Anal. calcd. for $C_{14}H_{28}O_4Si$: C, 58.30; H, 9.79; found C, 58.06; H, 9.60.

1,2-O-isopropylidene-3-t-butyidimethylsilyloxyltetradec-5-ene ***12***. Butyl lithium (34 ml, 0.085 mol, 2.5 M solution in hexane) was added to nonyltriphenylphosphonium bromide (40 g, 0.085 mol) in THF (200 ml) under Ar at 0°C and stirred for 1 hr. The mixture was cooled to −78°C, and a solution of **11** (22 g, 0.076 mol) in THF (50 ml) was added over 1 hr. The mixture was warmed to room temperature and stirred 1 hr. Water (300 ml) was added, and the mixture was extracted with ether/hexane (5 × 200 ml). Organic extracts were washed, dried, and concentrated as usual, and the residue was chromatographed on silica gel to yield **12** (27 g, 88%). IR (film): 2928, 2856, 1620, 1464, 1373, 1253, 1212, 1103, 834 cm^{-1}. CIMS: first isomer: 399 (M^++1, 31), 343 (12), 341 (100), 323 (38), 297 (3), 283 (30), 267 (51), 245 (21), 209 (62), 191(37), 175 (4), 165 (3), 151 (3), 133 (10), 113 (8), 101 (30). Second isomer: 399 (M^++1, 35), 383 (5), 343 (25), 341 (100), 323 (38), 297 (6), 283 (30), 267 (51), 245 (21), 209 (62), 191 (39), 171 (4), 133 (9), 111 (4), 101 (17). Anal. calcd. for $C_{23}H_{46}O_3Si$: C, 69.29; H, 11.64; found C, 69.43; H, 11.44.

(2R,3R)-1,2-O-Isopropylidene-3-t-butyldimethylsilyloxytetradecane ***13***. A mixture of **12** (13 g, 0.032 mol) and 10% Pd/C (1 g) in hexane (100 ml) was

hydrogenated under a slight positive pressure of H_2. After completion of the reaction, the mixture was filtered and concentrated to yield **13** (13 g, 99%); $[a]_D^{24}$ $+23.9^\circ$ (c 2.0, $CHCl_3$). IR (film): 2927, 2855, 1464, 1373, 1253, 1213, 1005 cm^{-1}. ^1H NMR ($CDCl_3$): 0.12 (s, 6H, $Si(C\underline{H}_3)_2$), 0.87–0.88 (m, 12H, $Si(C(C\underline{H}_3)_3)$ + H-14), 1.25–1.30 (m, 20H), 1.33 (s, 3H, —O—$C(C\underline{H}_3)_2$—O—), 1.40 (s, 3H, —O—$C(C\underline{H}_3)_2$—O—), 3.67 (ddd, 1H, $J = 7.3$, 6.5, 6.5 Hz, H-2), 3.70 (dd, 1H, $J = 8.1$, 7.3 Hz, H′-1), 3.92 (dd, 1H, $J = 8.1$, 6.5 Hz, H″-1), 4.03 (td, 1H, $J = 6.5$, 6.5 Hz, H-3). ^{13}C NMR ($CDCl_3$): 109.03, 78.90, 73.39, 65.64, 32.56, 31.89, 29.80, 29.72, 29.60, 29.55, 29.53, 29.29, 26.46, 25.92, 25.85, 25.57, 25.27, 22.63, 14.00, −4.23, −4.65. CIMS: 401 (M^++1, 51), 385 (8), 344 (24), 343 (100), 327 (8), 299 (16), 285 (30), 269 (2), 211 (25), 193 (9), 175 (9), 153 (3), 133 (7), 115 (5), 101 (13). Anal. calcd. for $C_{23}H_{48}O_3Si$: C, 68.94; H, 12.08; found C, 69.03; H, 12.30.

(2R,3R)-Tetradec-1,2,3-triol ***14***. A mixture of **13** (25 g, 0.06 mol), and TFA (10 ml) in 20% aqueous THF (100 ml) was refluxed overnight at 70°C. The mixture was neutralized with NH_4OH solution (10 ml 17 M), concentrated, and the residue was dissolved in methanol (500 ml), filtered, and concentrated to dryness. The residue was crystallized with ether/hexane to yield **14** (14 g, 92%); $[a]_D^{24}$ $+14.9^\circ$ (c 2.0, MeOH); m.p.: 65.0–66.0°C. IR (KBr): 3378, 2923, 2849, 1466, 1344, 1141, 1080, 1035 cm^{-1}. ^1H NMR ($CDCl_3$): 0.87 (t, 3H, $J = 7.0$ Hz, H-14), 1.20–1.55 (m, 20H), 2.55 (dd, 1H, $J = 6.4$, 5.2 Hz, —O$\underline{H}$—1), 2.11 (d, 1H, $J = 5.1$ Hz, —O$\underline{H}$—2), 2.59 (d, 1H, $J = 5.6$ Hz, —$\underline{OH}$—3), 3.53 (dddd, 1H, $J = 5.2$, 5.1, 4.1, 3.6 Hz, H-2,), 3.65 (tdd, 1H, $J = 6.5$, 5.6, 4.1 Hz, H-3), 3.68 (ddd, 1H, $J = 11.4$, 5.2, 5.2 Hz, H′-1), 3.77 (ddd, 1H, $J = 11.4$, 6.4, 3.6 Hz, H″-1). ^{13}C NMR ($CDCl_3$): 73.50, 72.84, 65.06, 33.77, 31.90, 29.58, 29.57, 29.32, 25.53, 22.66, 14.07. CIMS: 247 (M^++1, 79), 230 (13), 229 (100), 211 (13), 193 (5), 185 (2), 166 (3), 137 (2), 123 (2), 109 (2). Anal. calcd. for $C_{14}H_{30}O_3$: C, 68.25; H, 12.27; found C, 68.36; H, 12.14.

(2R,3R)-2,3-Dihydroxytetradecane-1-p-toluenesulfonate ***15***. *p*-TsCl (11 g, 0.058 mol) was added to a mixture of **14** (13 g, 0.053 mol) in pyridine (45 ml) at 0°C over 1 hr, and the mixture was stirred at 0°C overnight. After quenching with H_2O and extracting with ether (5 × 150 ml), the organic layer was washed, dried, and concentrated as usual, and the residue was recrystallized from ether/hexane to give **15** (13.8 g, 65%); $[a]_D^{24}$ $+11.2^\circ$ (c 1.0, $CHCl_3$); m.p.: 77.5–78.0°C. IR (KBr): 3412, 2920, 2849, 1598, 1464, 1361, 1179, 1098, 1075, 882, 817, 784, 664 cm^{-1}. ^1H NMR ($CDCl_3$): 0.87 (t, 3H, $J = 7.0$ Hz, H-14), 1.20–1.55 (m, 20H), 1.93 (d, 1H, $J = 6.1$ Hz, —O$\underline{H}$—3), 2.38 (d, 1H, $J = 5.9$ Hz, —OH—2), 2.45 (s, 3H, —Ar—$C\underline{H}_3$), 3.58 (tdd, 1H, $J = 7.1$, 6.1, 3.5 Hz, H-3), 3.71 (dddd, 1H, $J = 6.6$, 5.9, 4.6, 3.5 Hz, H-2), 4.05 (dd, 1H, $J = 10.4$, 6.6 Hz, H′-1), 4.11 (dd, 1H, $J = 10.4$, 4.6 Hz, H″-1), 7.36 (m, 2H, aromatics), 7.80 (m, 2H, aromatics). ^{13}C NMR ($CDCl_3$): 145.09, 132.87, 129.95, 127.98, 71.58, 71.42, 70.85, 33.51, 31.90, 29.62, 29.56, 29.52, 29.31, 25.52, 22.65, 21.60, 14.04. CIMS: 401 (M^++1,

9), 345 (12), 247 (13), 229 (54), 211 (18), 193 (13), 173 (100), 155 (7). Anal. calcd. for $C_{21}H_{36}O_5S$: C, 62.96; H, 9.07; found C, 62.87; H, 9.01.

*(2R,3R)-1,2-Epoxytetradecan-3-ol **16***. K_2CO_3 (4.5 g, 0.032 mol) was added to a solution of **15** (13 g, 0.032 mol) in methanol (75 ml), and the mixture was stirred at room temperature for 45 min. Ether/hexane (1:1) (200 ml) and H_2O (300 ml) were added, the organic layer was separated, and the aqueous layer was extracted with ether/hexane (3 × 100 ml). The combined organic extracts were washed with H_2O (2 × 100 ml) and saturated NaCl (200 ml), dried, and concentrated. The residue was purified by silica gel chromatography (ethyl acetate:hexane—2:1) and recrystallization from ether/hexane to give **16** (7 g, 94%); $[a]_D^{24}$ −2.8° (c 5.0, $CHCl_3$); m.p.: 40.0–41.0°C. IR (KBr): 3340, 2911, 2850, 1407, 1343, 1138, 1071 cm^{-1}. ^1H NMR ($CDCl_3$): 0.87 (t, 3H, $J = 7.0$ Hz, H-14), 1.20–1.55 (m, 18H), 1.52–1.63 (m, 2H, H-4) 1.76 (d, 1H, $J = 6.0$ Hz, —OH—3), 2.71 (dd, 1H, $J = 4.9$, 2.8 Hz, H′-1), 2.81 (dd, 1H, $J = 4.9$, 4.1 Hz, H″-1), 2.98 (ddd, 1H, $J = 5.0$, 4.1, 2.8 Hz, H-2), 3.43 (dddd, 1H, $J = 6.8$, 6.4, 6.0, 5.0 Hz, H-3). ^{13}C NMR ($CDCl_3$): 71.66, 55.30, 45.05, 34.47, 31.88, 29.59, 29.54, 29.50, 29.29, 25.27, 22.63, 14.00. CIMS: 229 (M^++1, 100), 211 (30), 199 (6), 183 (5), 113 (4). Anal. calcd. for $C_{14}H_{28}O_2$: C, 73.62; H, 12.37; found C, 73.48; H, 12.19.

*(2R,3R)-1,2-Epoxy-3-t-butyldimethylsilyloxytetradecane **17***. The procedure was analogous to that described for **10**, substituting **16** (3.4 g, 0.015 mol) for **9**, with other reagents used in proportional amounts. The crude product was chromatographed on silica gel (ether:hexane—1:9) to produce **17** (4.7 g, 91%); $[a]_D^{24}$ +5.3° (c 1.5, $CHCl_3$). IR (film): 2927, 2855, 1413, 1253, 837 cm^{-1}. ^1H NMR ($CDCl_3$): 0.05 (s, 3H, $SiC\underline{H}_3$), 0.12 (s, 3H, $SiCH_3$), 0.85–0.90 (m, 12H, $SiC(CH_3)_3$ + H-14), 1.20–1.55 (m, 20H), 2.55 (dd, 1H, $J = 4.9$, 2.8 Hz, H′-1), 2.75 (dd, 1H, $J = 4.9$, 4.2 Hz, H″-1), 2.91 (ddd, 1H, $J = 6.6$, 4.2, 2.8 Hz, H-2), 3.25 (ddd, 1H, $J = 6.8$, 6.6, 5.9 Hz, H-3). ^{13}C NMR ($CDCl_3$): 74.65, 55.93, 44.78, 34.77, 31.89, 29.65, 29.61, 29.55, 29.51, 29.29, 25.87, 25.26, 22.63, 18.16, 14.00, −4.37, −4.98. CIMS: 343 (M^++1, 100), 327 (18), 313 (13), 299 (4), 285 (91), 211 (45), 193 (22), 175 (23), 133 (13), 111 (10). Anal. calcd. for $C_{21}H_{42}O_2Si$: C, 71.13; H, 11.95; found C, 70.86; H, 11.71.

*(2R,3R)-1,2-Epoxytetradecane-3-p-toluenesulfonate **22***. A mixture of **16** (3.4 g, 0.015 mol), *p*-TsCl (3.15 g, 0.016 mol), and pyridine (14 ml) was stirred overnight at 0°C. The mixture was quenched with H_2O (150 ml) and extracted with ether (4 × 100 ml). The combined organic extracts were washed, dried, and concentrated as usual, and the residue was chromatographed on silica gel (ether:hexane—1:1). Recrystallization from ether/hexane yielded **22** (4.2 g, 73.6%); $[a]_D^{24}$ −13° (c 1.0, $CHCl_3$); m.p.: 67.5–68.0°C. IR (KBr): 2924, 2851, 1598, 1463, 1356, 1178, 1095, 1042, 843 cm^{-1}. ^1H NMR ($CDCl_3$): 0.87 (t, 3H, $J = 7.0$ Hz, H-14), 1.17-1.35 (m, 18H), 1.60–1.75 (m, 2H, H-4), 2.45 (s, 3H, Ar—$C\underline{H}_3$), 2.63 (dd, 1H, $J = 4.8$, 2.6 Hz, H′-1), 2.77 (1H, $J = 4.8$, 4.2 Hz, H″-1), 3.05 (ddd, 1H, $J = 6.1$, 4.2, 2.6 Hz, H-2), 4.33 (ddd, 1H, $J = 7.4$, 6.1, 5.9 Hz, H-3), 7.36 (m, 2H,

aromatics), 7.81 (m, 2H, aromatics). ^{13}C NMR ($CDCl_3$): 144.51, 134.59, 129.60, 127.88, 83.24, 52.66, 44.73, 31.89, 31.83, 29.57, 29.44, 29.30, 29.22, 24.81, 22.64, 21.57, 14.01. CIMS: 383 (M^++1, 12), 229 (7), 212 (11), 211 (77), 195 (30), 193 (30), 177 (52), 133 (100), 111 (9), 109 (9), 105 (10). Anal. calcd. for $C_{21}H_{34}O_4S$: C, 65.93; H, 8.97; found C, 65.95; H, 8.88.

(5R,6R)-1,6-Di-t-butyldimethylsiloxy-5-hydroxyheptadec-2-yne ***18***. Butyllithium (2.5 M in hexane, 9.6 ml, 0.024 mol) was added to 1-*t*-butyldimethylsiloxy-2-propyne (4.3 g, 0.025 mol) in THF (20 ml) at −78°C under Ar. After stirring for 45 min, BF_3–Et_2O (2.9 ml 0.024 mol) was added, and stirring was continued for 45 min. A solution of **17** (4.1 g, 0.012 mol) in THF (7 ml) was added, and the mixture was stirred for 2 hr at −78°C. The cooling bath was removed and saturated NH_4Cl (100 ml) was added. The aqueous solution was extracted with 50% ether/hexane (4 × 100 ml), and the combined organic extracts were washed, dried, and concentrated as usual. The residue was chromatographed on silica gel (ether: hexane—1:3) to yield **18** (5.3 g, 87%); $[a]_D^{24}$ −11.9° (c 2.0, $CHCl_3$). IR (film): 3554, 2928, 2856, 1466, 1365, 1254, 1139, 1079, 1003, 938 cm^{-1}, ^{1}H NMR ($CDCl_3$): 0.10–0.12 (m, 12H, 2 Si(CH_3)$_2$), 0.80–0.90 (m, 21H, 2 Si(C($C\underline{H}_3$)$_3$) + H-17), 1.20–1.30 (m, 18H), 1.39–1.49 (m, 1H, H′-7), 1.55–1.70 (m, 1H, H″-7), 2.35 (ddt, 1H, J = 16.5, 7.7, 2.1 Hz, H′-4), 2.42 (ddt, 1H, J = 16.5, 6.1, 2.1 Hz H″-4), 3.62 (ddd, 1H, J = 7.7, 6.1, 2.2 Hz, H-5), 3.75 (ddd, 1H, J = 7.7, 2.2 Hz, H-6), 4.30 (t, 2H, J = 2.1 Hz, H-1). ^{13}C NMR ($CDCl_3$): 81.68, 80.50, 73.05, 71.29, 51.86, 33.83, 31.89, 29.77, 29.62, 29.60, 29.55, 29.30, 25.89, 25.83, 25.24, 24.54, 22.63, 18.21, 18.08, 14.01, −4.20, −4.62, −5.13. CIMS: 513 (M^++1, 80), 512 (M^+, 2), 495 (5), 455 (11), 397 (12), 381 (13), 363 (4), 343 (13), 287 (93), 249 (20), 229 (23), 187 (6), 171 (62), 155 (9), 143 (10), 133 (100), 123 (60). Anal. calcd. for $C_{29}H_{60}O_3Si_2$: C, 67.91; H, 11.80; found C, 67.75; H, 11.60.

(5R,6R)-1,6-Di-t-butyldimethylsiloxy-heptadec-2-yn-5-p-toluenesulfonate ***19***. A mixture of **18** (5.1 g, 0.01 mol) and *p*-TsCl (2.3 g, 0.012 mol) in pyridine (14 ml) was stirred at 0°C for 48 hr. The mixture was quenched with H_2O (200 ml) with stirring, and then extracted with ether (4 × 150 ml), and the organic extracts were washed, dried, and concentrated as usual. Chromatography on silica gel (ether:hexane—1:9) followed by crystallization from ether/hexane gave **19** (5.6 g, 84.8%); $[a]_D^{24}$ +14.5° (c 2.0, $CHCl_3$); m.p.: 50.5–51.5°C. IR (KBr): 2928, 2857, 1598, 1467, 1365, 1255, 1174, 1079, 992, 838 cm^{-1}. ^{1}H NMR ($CDCl_3$): 0.05–0.12 (m, 12H, 2 Si(CH_3)$_2$), 0.80–0.90 (m, 21H, 2 Si(C($C\underline{H}_3$)$_3$) + H-17), 1.10–1.35 (m, 18H), 1.40–1.55 (m, 2H, H-7), 2.43 (s, 3H, Ar—$C\underline{H}_3$), 2.45 (ddd, 1H, J = 17.1, 7.5, 2.0 Hz, H′-4), 2.67 (1H, H″-4, ddd, J = 17.1, 5.5, 2.1 Hz), 3.89 (1H, H-5, ddd, J = 7.5, 5.5, 3.5 Hz), 4.10 (2H, H-1, m), 4.43 (ddd, 1H, J = 7.4, 5.4, 3.5 Hz, H-6), 7.36 (m, 2H, aromatics), 7.81 (m, 2H, aromatics). ^{13}C NMR ($CDCl_3$): 144.45, 134.48, 129.61, 127.95, 81.88, 80.83, 80.26, 71.74, 51.72, 31.90, 31.54, 29.61, 29.57, 29.51, 29.31, 25.82, 25.54, 22.65, 21.55, 19.76, 18.26, 17.99, 14.02, −4.51, −4.55, −5.14. CIMS: 668 (M^++2, 28), 609 (4), 513 (8), 495

(22), 363 (22), 288 (22), 287 (100), 249 (30), 231 (30), 229 (20), 213 (16), 173 (21), 157 (96), 133 (60). Anal. calcd. for $C_{36}H_{66}O_5SSi_2$: C, 64.82; H, 9.98; found C, 64.99; H, 9.74.

*(5S,6R)-cis-5,6-Epoxyheptadec-2-yn-1-ol **20**.* A mixture of **19** (5.4 g, 0.008 mol) and $(n\text{-Bu})_4N^+F^-$ (1 M solution in THF, 20 ml, 0.02 mol) in THF (10 ml) was stirred overnight at room temperature. The mixture was added to H_2O (175 ml and extracted with 50% ether/hexane (4 × 150 ml). The organic extracts were washed with 10% HCl (v/v, 2 × 200 ml), 10% $NaHCO_3$ (w/v, 200 ml), saturated NaCl (200 ml), dried over anhyd. Na_2SO_4, and concentrated. The crude product was chromatographed on silica gel (ether:hexane—2:1) to yield **20** (1.4 g, 78%); $[a]_D^{24}$ +30.1° (c 2.0, $CHCl_3$); m.p.: 65.5–66.0°C; IR (KBr): 3368, 3250, 2922, 2851, 1418, 1170, 1133 cm^{-1}. ^1H NMR ($CDCl_3$) ppm: 0.87 (t, 3H, $J = 7.0$ Hz, H-17), 1.25–1.55 (m, 20H), 2.35 (ddt, 1H, $J = 17.1$, 6.8, 2.3 Hz, H′-4), 2.60 (ddt, 1H, $J = 17.1$, 5.8, 2.2 Hz, H″-4), 2.95 (ddd, 1H, $J = 6.2$, 5.6, 4.2 Hz, H-6), 3.15 (ddd, 1H, $J = 6.8$, 5.8, 4.1 Hz, H-5), 4.26 (dd, 2H, $J = 2.2$, 2.1 Hz, H-1). ^{13}C NMR ($CDCl_3$): 81.44, 80.43, 57.04, 54.90, 51.29, 31.90, 29.61, 29.53, 29.47, 29.30, 27.57, 26.50, 22.65, 18.86, 14.02. CIMS: 267 (M^++1, 16), 251 (17), 249 (100), 235 (5), 231 (31), 198 (11), 197 (89), 179 (11), 155 (7), 137 (12), 123 (14), 121 (11). Anal. calcd. for $C_{17}H_{30}O_2$: C, 76.63; H, 11.36; found C, 76.85; H, 11.25.

*(5R,6R)-1-t-Butyldimethylsiloxy-5-hydroxy-heptadec-2-yn-6-p-toluenesulfonate **23**.* The method was similar to that described for **18**. Butyllithium (2.5 M in hexane, 8 ml, 0.02 mol), 1-*t*-butyldimethylsiloxy-2-propyne (3.6 g, 0.021 mol) in THF (20 ml), BF_3–Et_2O (2.45 ml, 0.02 mol) and **22** (4.1 g, 0.01 mol) in THF (7 ml) were used for the reaction. After addition of **22**, the mixture was stirred for 5 hr at −78°C. The cooling bath was removed, and saturated NH_4Cl (100 ml) was added to the mixture. The aqueous solution was extracted with ether (4 × 100 ml) and the combined extracts were washed, dried, and concentrated as usual to yield **23**, which was used directly for the next step.

*(5R,6S)-cis-5,6-Epoxy-1-t-butyldimethylsiloxy-heptadec-2-yne **24**.* K_2CO_3 (2 g, 0.014 mol) was added to a solution of **23** in methanol (30 ml) and stirred for 1.5 hr at room temperature. A total of 50% ether/hexane (100 ml) and H_2O (200 ml) were added to the mixture, the organic layer was separated, and the aqueous layer was extracted with ether/hexane (3 × 100 ml). The combined organic extracts were washed with water and saturated NaCl, dried, and concentrated to yield **24** with 80% purity by GC, containing some unreacted 1-*t*-butyldimethylsiloxy-2-propyne. The crude product was used without purification.

*(5R,6S)-cis-5,6-Epoxyheptadec-2-yn-1-ol **25**.* $(n\text{-Bu})_4N^+F^-$ (1 M solution in THF, 10 ml, 0.01 mol) was added to a solution of crude **24** in THF (10 ml), and the mixture was stirred for 2 hr at room temperature. Aqueous workup and purification (identical to that described in **20**) yielded **25** (1.26 g, 47.3% from 23); $[a]_D^{24}$ −30.3° (c 2.0, $CHCl_3$); m.p.: 65.5–66.0°C. Anal. calcd. for $C_{17}H_{30}O_2$: C, 76.63; H, 11.36; found C, 76.85; H, 11.50. Other spectroscopic data were identical to **20**.

(Z)-2-(5S,6R)-cis-5,6-Epoxyheptadecen-1-ol ***21***. A solution of $NaBH_4$ (0.5 g) in NaOH (0.5 ml, 3 M) was slowly added to a solution of $Ni(OAc)_4$ (1 g) in methanol (20 ml) under H_2 at room temperature over 5 min. After stirring for 30 min, the solution was decanted, and residue was washed with methanol (2 × 10 ml). Methanol (15 ml), ethylenediamine (1 ml), and **20** (1.4 g, 0.005 mol) were added to the residue, and the mixture was stirred under H_2 for 25 min. The solution was decanted and residue was washed with ether (4 × 25 ml). The combined extracts were diluted with ether (250 ml) and washed with water, 5% HCl, 5% $NaHCO_3$, and saturated NaCl, dried, and concentrated. The crude product was chromatographed on silica gel (ether:hexane—2:1) to yield crystalline **21** (1.18 g, 84%); $[a]_D^{24}$ −9.9° (c 1.75, $CHCl_3$); m.p.: 44.5–45.0°C. IR (KBr): 3325, 2919, 2849, 1657, 1367, 1233, 1132, 716 cm^{-1}, ^1H NMR (C_6D_6): 0.96 (t, 3H, J = 7.0 Hz, H-17), 1.22 (dd, 1H, J = 6.3, 5.5 Hz, —O<u>H</u>-1), 1.25–1.53 (m,20H), 2.04 (ddd, 1H, J = 14.7, 7.7, 5.1 Hz, H′-4), 2.26 (ddd, 1H, J = 14.7, 7.7, 7.6 Hz, H″-4), 2.70–2.77 (m, 2H, H-5 + H-6), 3.96 (ddd, 1H, J = 18.5, 6.6, 6.3 Hz, H′-1), 4.01 (ddd, 1H, J = 18.5, 6.6, 5.5 Hz, H″-1) 5.50 (dtt, 1H, J = 10.9, 7.7, 1.4 Hz, H-3), 5.72 (dtt, 1H, J = 10.9, 6.6, 1.5 Hz, H-2). ^{13}C NMR (C_6D_6): 132.34, 126.87, 58.39, 57.36, 55.88, 32.29, 30.06, 29.96, 29.82, 29.76, 28.13, 26.98, 26.65, 23.07, 14.30. CIMS: 269 (M^++1, 9), 267 (3), 253 (2), 252 (18), 251 (100), 233 (10), 229 (3), 215 (5), 198 (8), 197 (58), 183 (12). Anal. calcd. for $C_{17}H_{32}O_2$: C, 70.05; H, 12.02; found C, 70.16; H, 11.78.

(Z)-2-(5R,6S)-cis-5,6-Epoxyheptadecen-1-ol ***26***. The method was identical to that described for the preparation of **21**, and **25** (1.2 g, 0.0045 mol) was used to yield **26** (1.07 g, 89%); $[a]_D^{24}$ +9.6° (c 1.75, $CHCl_3$); m.p.: 44.5–45.0°C. Anal. calcd. for $C_{17}H_{32}O_2$: C, 70.05; H, 12.02; found C, 70.12; H, 12.30. Other spectroscopic data were identical to **21**.

(2S,3R,5S,6R)-cis-2,3-cis-5,6-Diepoxyheptadecanol ***27***. (+)-(L)-Diethyl tartrate (0.35 ml, 0.002 mol) was added to a solution of $Ti(OCH(CH_3)_2)_4$ (0.59 ml, 0.002 mol) in CH_2Cl_2 (10 ml) at −23°C under Ar, and the mixture was stirred 15 min. A solution of **21** (0.5 g, 0.002 mol) in CH_2Cl_2 (1 ml) was added to the mixture. After stirring for 10 min, *t*-butylhydroperoxide (3.3 M solution in toluene, 1.2 ml, 0.004 mol) was added. The mixture was kept at −20°C for 40 hr, and then the temperature was increased to −10°C and 10% aq. tartaric acid (15 ml) was added. The mixture was stirred at −10°C for 20 min and then 30 min at rt, until both layers became clear. The organic layer was separated, and the aqueous layer was extracted with CH_2Cl_2 (3 × 50 ml). The combined organic extracts were washed with 10% NaOH, water, 10% HCl, 10% $NaHCO_3$, and saturated NaCl, dried, and concentrated. The crude product was chromatographed on silica gel (ethyl acetate:hexane—1:1) to yield crystalline **27** (0.38 g, 66.6%); $[a]_D^{24}$ +6° (c 2.5, $CHCl_3$); m.p.: 48.5–49.5°C. IR (KBr): 3347, 2920, 2851, 1468, 1381, 1319, 1040, 953, 849 cm^{-1}. ^1H NMR ($CDCl_3$): 0.89 (t, 3H, J = 7.0 Hz, H-17), 1.20–1.55 (m, 20H), 1.69 (dd, 1H, J = 6.2, 5.7 Hz, —OH-1), 1.86–1.89 (m, 2H, H-4),

2.96 (ddd, 1H, $J = 6.1, 5.8, 4.2$ Hz, H-6), 3.08 (ddd, 1H, $J = 6.4, 5.8, 4.2$ Hz, H-5), 3.17–3.22 (m, 2H, H-2 + H-3), 3.77 (ddd, 1H, $J = 11.2, 5.7, 5.7$ Hz, H′-1), 3.87 (ddd, 1H, $J = 11.2, 6.2, 4.3$ Hz). ^{13}C NMR ($CDCI_3$): 60.55, 56.74, 56.10, 54.33, 54.09, 31.88, 29.59, 29.51, 29.47, 29.30, 27.87, 27.07, 26.55, 22.65, 14.06, CIMS: 286 (4), 285 (M^++1, 22), 269 (3), 268 (13), 267 (100), 265 (2), 249 (20), 231 (12), 224 (7), 223 (4), 221 (6), 197 (33), 183 (7), 179 (5), 155 (1), 141 (1), 113 (5). Anal. calcd. for $C_{17}H_{32}O_3$: C, 71.77; H, 11.35; found C, 71.54; H, 11.42.

(2R,3S,5S,6R)-cis-2, 3-cis-5, 6-Diepoxyheptadecanol ***29***. The method and quantities were identical to those reported to prepare **27**, except that (−)-(D)-diethyl tartrate was used instead of (+)-(L)-DET to produce **29** (0.41 g, 71.9%); $[a]_D^{24}$ −21.9° (c 2.5, $CHCl_3$); m.p.: 85.5–86.0°C. IR (KBr): 3280, 2916, 2849, 1468, 1392, 1278, 1164, 1123, 1057, 914 cm^{-1}. ^{1}H NMR ($CDCl_3$): 0.87 (t, 3H, $J = 7.0$ Hz, H-17), 1.20–1.35 (m, 18H), 1.40 (ddd, 1H, $J = 14, 4, 10, 8.5$ Hz, H′-4), 1.45–1.55 (m, 2H, H-7), 2.27 (ddd, 1H, $J = 14.4, 4.9, 2.9$ Hz, H″-4), 2.91–2.98 (m, 1H, —OH-1), 3.09 (ddd, 1H, $J = 10.9, 6.6, 4.4$ Hz, H-6), 3.15 (ddd, 1H, $J = 10, 4.4, 2.9$ Hz, H-5), 3.20–3.27 (m, 2H, H-2 + H-3), 3.41–3.50 (m, 1H, H′-1), 3.85–3.97 (m, 1H, H″-1). ^{13}C NMR ($CDCl_3$): 60.03, 58.03, 55.39, 54.11, 31.90, 29.61, 29.51, 29.41, 29.32, 27.82, 26.70, 26.33, 22.67, 14.08. CIMS: 286 (18), 285 (M^++1, 100), 283 (2), 269 (1), 268 (10), 267 (54), 265 (2), 249 (15), 237 (2), 231 (10), 224 (1), 223 (5), 221 (2), 197 (16), 195 (2), 183 (4), 179 (4), 155 (1), 141 (2), 113 (3). Anal. calcd. for $C_{17}H_{32}O_3$: C, 71.77; H, 11.35; found C, 71.95; H, 11.60.

(2S,3R,5R,6S)-cis-2,3-cis-5,6-Diepoxyheptadecanol ***33***. The method and quantities were identical to those reported to prepare **27**. The allylic epoxy alcohol **26** and (+)-(L)-diethyl tartrate were used to produce **33** (0.42 g, 73.6%); $[a]_D^{24}$ +20.9° (c 2.5, $CHCl_3$); m.p.: 85.5–86.0°C. Anal. calcd. for $C_{17}H_{32}O_3$: C, 71.77; H, 11.35; found C, 71.49; H, 11.37. Other spectroscopic data were identical to **29**.

(2R,3S,5R,6S)-cis-2,3-cis-5,6-Diepoxyheptadecanol ***31***.. The method and quantities were identical to those reported to prepare **27**. The allylic epoxy alcohol **26** and (−)-(D)-diethyl tartrate were used to produce **31** (0.39 g, 68.4%); $[a]_D^{24}$ −6.9° (c 2.5, $CHCl_3$); m.p.: 49.5–50.5°C. Anal. calcd. for $C_{17}H_{32}O_3$: C, 71.77; H, 11.35; found C, 71.49; H, 11.14. Other spectroscopic data were identical to **27**.

(2S,3R,5S,6R)-cis-2,3-cis-5,6-Diepoxyheptadecane-1-p-toluenesulfonate ***28***. *p*-TsCl (0.3 g, 0.0015 mol) was added to a mixture of **27** (0.35 g, 0.0012 mol) in pyridine (2 ml), and the mixture was stirred overnight at 0°C. Following addition of H_2O (15 ml), the mixture was extracted with ether (3 × 25 mL). The organic extracts were washed with 5% HCl, 10% $NaHCO_3$, and saturated NaCl, dried, and filtered through a silica gel bed. After concentration, crude **28** was used without further purification.

(2R,3S,5S,6R)-cis-2,3-cis-5,6-Diepoxyheptadecane-1-p-toluenesulfonate ***30***. The method and quantities were identical to those described in **28**. Compound **29** was used instead of **27** to yield **30**.

(2S,3R,5R,6S)-cis-2, 3-cis-5, 6-Diepoxyheptadecane-1-p-toluenesulfonate ***34***. The method and quantities were identical to those described in **28**. Compound **33** was used instead of **27** to yield **34**.

(2R,3S,5R,6S)-cis-2,3-cis-5,6-Diepoxyheptadecane-1-p-toluenesulfonate ***32***. The method and quantities were identical to those described in **28**. Compound **31** was used instead of **27** to yield **32**.

(Z)-3-(6S,7R,9S,10R)-cis-6,7-cis-9,10-Diepoxyhenicosene ***1***. *t*-Butyllithium (1.5 M solution in hexane, 8 ml, 0.012 mol) was added to a solution of 1-iodo-(*Z*)-1-butene (1 g, 0.006 mol) in THF (5 ml) at 0°C under Ar, and the mixture was stirred for 2 hr, and then transferred by canula to a suspension of CuI (0.57 g, 0.003 mol) in THF (5 ml) at −50°C under Ar. The temperature was gradually increased to −20°C. After sirring for 1.5 h, HMPA (1 ml), a solution of **28** (∼0.0012 mol) in THF (1 ml), and triethyl phosphite (0.5 ml) were added to the reaction mixture. The mixture was stirred for 12 hr, and the temperature was allowed to reach room temperature. The reaction mixture was quenched with aq. NH_4Cl (25 ml) and extracted with ether (5 × 50 ml). The combined organic extract was washed, dried, and concentrated as usual. The residue was purified by silica gel chromatography followed by HPLC (detector 210 nm, Nova-pak^{TM} silica 3.9 × 150 mm column with 1 ml/min 3% ether/hexane flow) to yield 1 with >99.9% de by GC (0.041 g, 10.8%); $[a]_D^{24}$ +4.9° (c 1.61, hexane); [lit. Muto and Mori, 2003; $[a]_D^{24}$ +5.0° (c 0.55, $CHCl_3$)]. IR, ^{1}H NMR, and ^{13}C NMR were essentially identical to those reported. Anal. calcd. for $C_{21}H_{38}O_2$: C, 78.19; H, 11.88; found C, 78.06; H, 11.69.

(Z)-3-(6R,7S,9S,10R)-cis-6,7-cis-9,10-Diepoxyhenicosene ***2***. The method used was identical to that described for 1. Compound **30** was used instead of **28** to yield **2** with >99.9% de by GC (0.068 g, 17.9%). $[a]_D^{24}$ −9.3° (c 1.07, hexane); m.p.: 39–40°C; [lit. Muto and Mori, 2003; $[a]_D^{24}$ −21.3° (c 0.50, $CHCl_3$); m.p.: 44–45°C]. IR, ^{1}H NMR, and ^{13}C NMR were essentially identical to those reported. Anal. calcd. for $C_{21}H_{38}O_2$: C, 78.19; H, 11.88; found C, 77.98; H, 11.69.

(Z)-3-(6S,7R,9R,10S)-cis-6,7-cis-9,10-Diepoxyhenicosene ***4***. The method used was identical to that described for **1**. Compound **34** was used instead of **28** to yield **4** with >99.9% de by GC (0.048 g, 12.6%); $[a]_D^{24}$ +9.1° (c 0.77, hexane); m.p.: 39–40°C; [lit. Muto and Mori, 2003; $[a]_D^{24}$ +21.4° (c 0.41, $CHCl_3$); m.p.: 44–45°C]. IR, ^{1}H NMR, and ^{13}C NMR were identical to **2**. Anal. calcd. for $C_{21}H_{38}O_2$: C, 78.19; H, 11.88; found C, 78.06; H, 11.89.

(Z)-3-(6R,7S,9R,10S)-cis-6,7-cis-9,10-Diepoxyhenicosene ***3***. The method used was identical to that described for **1**. Compound **32** was used instead of **28** to yield **3** with >99.9% de by GC (0.054 g, 14.2%); $[a]_D^{24}$ −4.8° (c 1.85, hexane); [lit. Muto and Mori, 2003; $[a]_D^{24}$ −4.5° (c 0.55, $CHCl_3$)]. Anal. calcd. for $C_{21}H_{38}O_2$: C, 78.19; H, 11.88; found C, 77.95; H, 11.71]. IR, ^{1}H NMR, and ^{13}C NMR were identical to **1**.

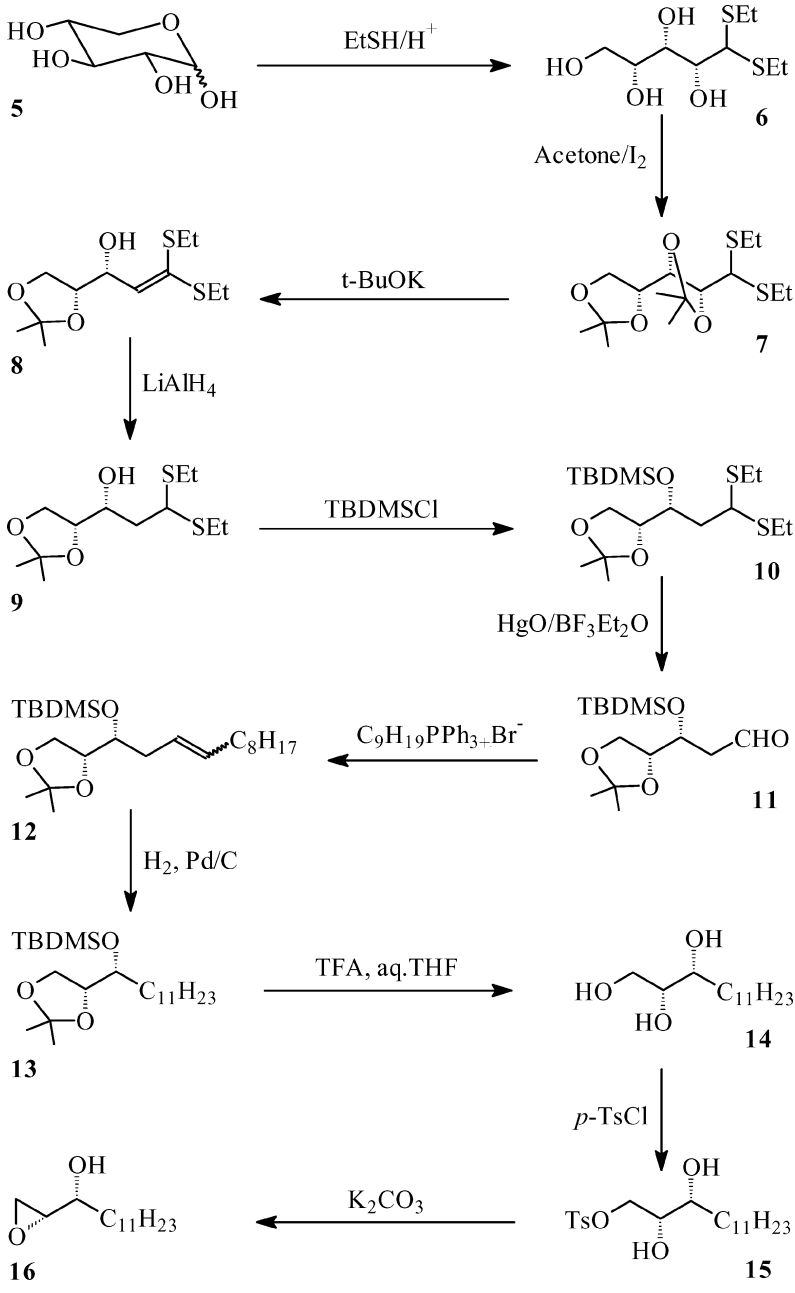
RESULTS AND DISCUSSION

Our synthetic strategy is outlined in Schemes 1–4. Synthesis was initiated using chirally pure D-xylose **5**. The hydroxyl groups at C-3 and C-4 of D-xylose were utilized for the construction of the 9,10-epoxy group in all optical isomers of the (*Z*)-3-*cis*-6,7-*cis*-9,10-diepoxyhenicosenes. D-xylose was converted to **9** as described in the synthesis of (*Z*)-6-(9*S*,10*R*)-*cis*-9,10-epoxyhenicosene, the sex pheromone of the tiger moth, *Phragmatobia fuliginosa* (Rollin and Pougny, 1986), and (*Z*, *Z*)-3,6-(9*S*,10*R*)-*cis*-9,10-epoxyhenicosadiene, the pheromone of salt marsh caterpillar, *Estigmene acrea* (Pougny and Rollin, 1987).

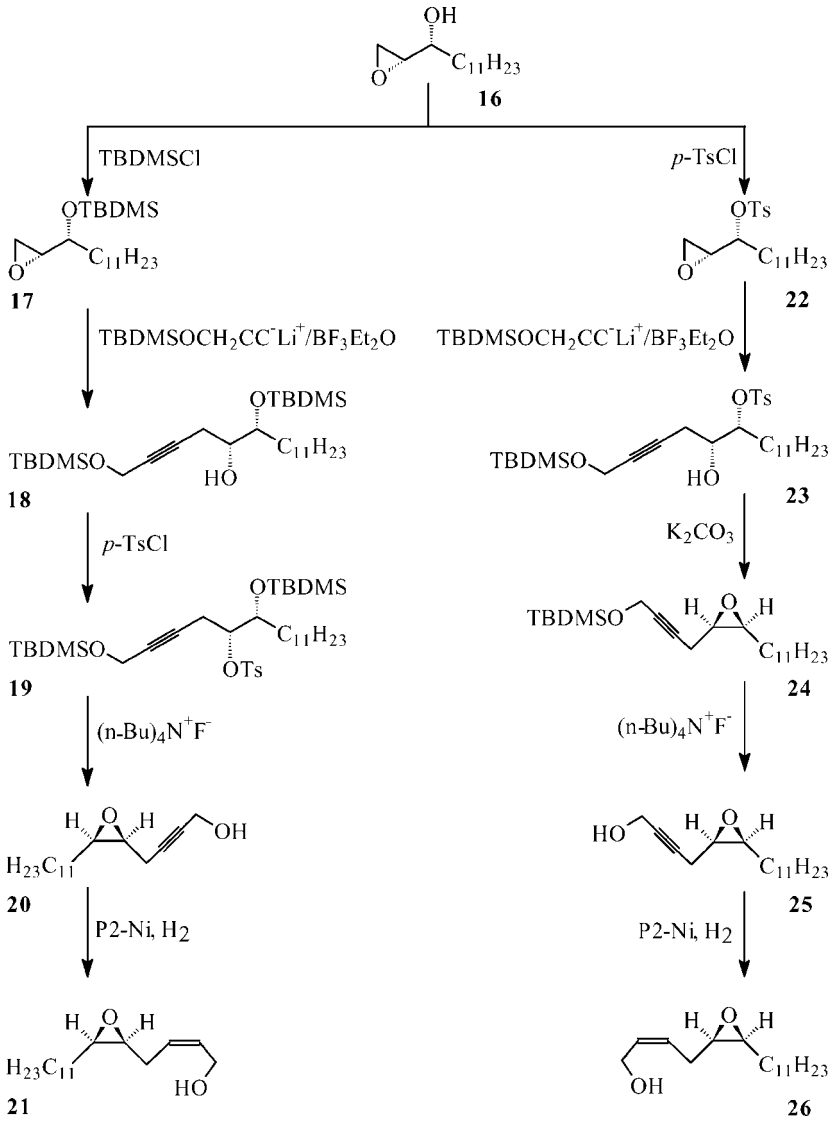
Reaction conditions and reagents used for selected steps were modified in our synthetic route to **9** in the following manner (Scheme 1). Conversion of D-xylose to ethanedithioacetal **6** was carried out at 0°C to minimize evaporation of ethanethiol during the exothermic acetal formation reaction. The iodine-catalyzed isopropylidenation methodology described by Kartha (1986) was used for the preparation of diacetal **7** in high yield, without apparent formation of other possible isomers. Compound **7** was converted to ketene dithioacetal **8** as previously reported and was reduced using 1 molar equivalent of $LiAlH_4$ and **8** to form **9**. Although removal of dithioacetal groups with Hg^{2+} ions is typical (Rollin and Pougny, 1986), this method gave low yields with compound **9** probably due to the high solubility of the aldehyde product in water. Therefore, the hydroxyl group in compound **9** was first converted to TBDMS ether **10**, which was then converted to the corresponding aldehyde **11** in high yield and purity. Wittig coupling of **11** with nonyltriphenylphosphonium bromide gave **12**, which was hydrogenated over Pd/C to form **13**. Because the presence of very small quantities of impurity were found to completely inhibit hydrogenation, compound **12** was carefully chromatographed on silica gel prior to hydrogenation.

Deprotection of both isopropylidene and TBDMS groups in compound **13** with TFA in aq. THF yielded (2*R*, 3*R*)-tetradeca-1,2,3-triol **14**. Selective tosylation of **14** was carried out with 1 equivalent of TsCl at 0°C to produce the monotosylate, **15**, which was purified by recrystallization. Treatment of tosylate **15** with methanolic K_2CO_3 produced the key intermediate (2*R*, 3*R*)-1,2-epoxytetradecan-3-ol **16** (Bell and Ciaccio, 1993). As outlined in Scheme 2, epoxy alcohol **16** was used for the synthesis of both intermediate epoxy allylic alcohols, i.e., enantiomers of **21** and **26**.

The formation of epoxy allylic alcohol **21** began with protection of the hydroxyl group of **16** with TBDMSCl to form **17**. The oxirane end of **17** was then coupled with a TBDMS protected propynylborane to form **18** (Yamaguchi and Hirao, 1983). The hydroxyl group at C-5 of **18** was tosylated to form the ester **19** and stereospecifically cyclized to form **20** with (5*S*, 6*R*) configuration (Bell and Ciaccio, 1993). Because this cyclization followed an S_N2 mechanism, it produced



Scheme 1. Conversion of D-xylose into chiral intermediate **16**.



Scheme 2. Divergent syntheses of **21** and **26**.

a single enantiomer without any racemization. The triple bond of **20** was then reduced with P2–Ni to form the epoxy *Z*-allylic alcohol **21**.

To produce the antipode of **21**, epoxy alcohol **16** was converted to tosylate **22**, which was coupled with TBDMS-protected propynylborane to form **23**, as in the

synthesis of **18**. Treatment of **23** with K_2CO_3 gave **24** with (5*R*, 6*S*) configuration. Removal of the TBDMS group at C-1 of **24** produced **25**, the antipode of **20**. Reduction of **25** with P2–Ni gave the intermediate epoxy allylic alcohol **26**.

Following the syntheses of chiral **21** and **26**, the next step was to establish the 2,3-epoxy group in each alcohol with proper stereochemistry. We utilized the diethyl tartrate (DET) mediated epoxidation (Katsuki and Sharpless, 1980; Rossiter et al., 1981; Hanson and Sharpless, 1986). As shown in Figure 2, epoxidation of **21** with (+)-(L)-DET and (−)-(D)-DET produced (2*S*, 3*R*, 5*S*, 6*R*)-*cis*-2,3-*cis*-5,6-diepoxyheptadecanol **27** and (2*R*, 3*S*, 5*S*, 6*R*)-*cis*-2,3-*cis*-5,6-diepoxyheptadecanol **29**, respectively. Similarly **26** with (−)-(D)-DET and (+)-(L)-DET yielded (2*R*, 3*S*, 5*R*, 6*S*)-*cis*-2,3-*cis*-5,6-diepoxyheptadecanol **31** and (2*S*, 3*R*, 5*R*, 6*S*)-*cis*-2,3-*cis*-5,6-diepoxyheptadecanol **33**, respectively (see Figure 2).

Although the Katsuki–Sharpless asymmetric epoxidation results in products contaminated with small amounts of enantiomer, in this application, it was suitable for the preparation of **27**, **29**, **31**, and **33**. The presence of the chirally pure

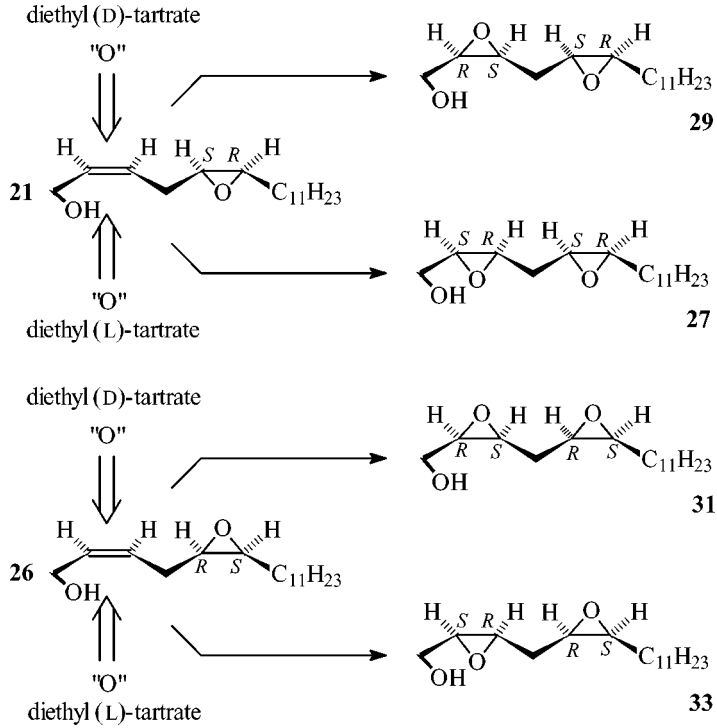
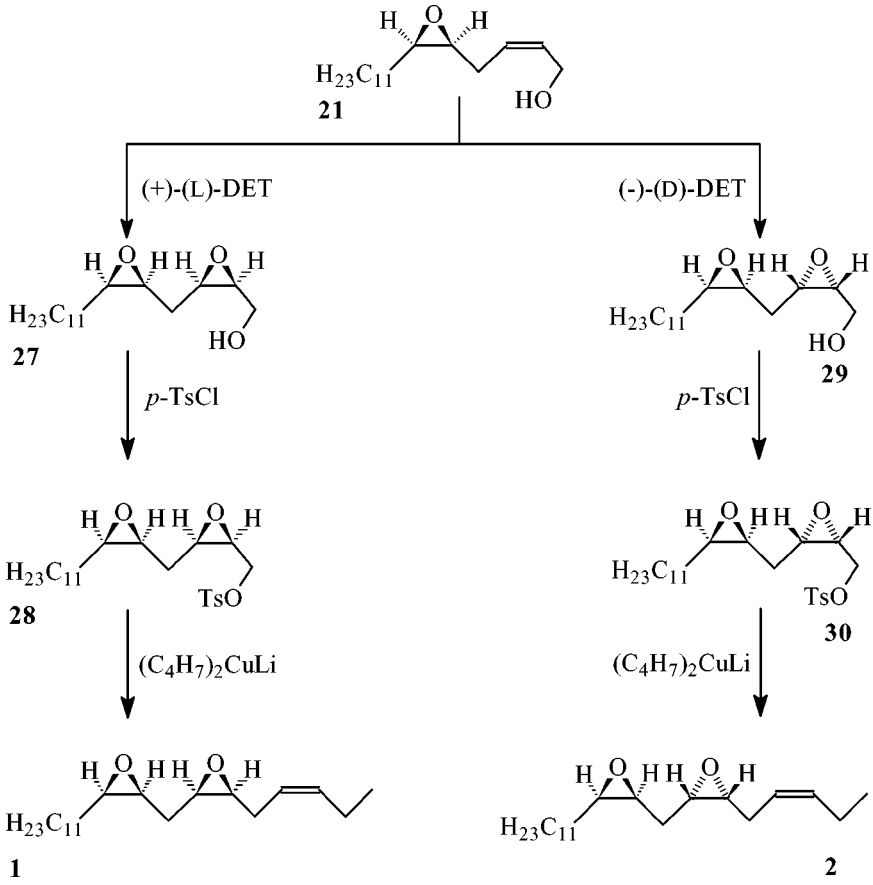


FIG. 2. Katsuki–Sharpless epoxidations of **21** and **26**.

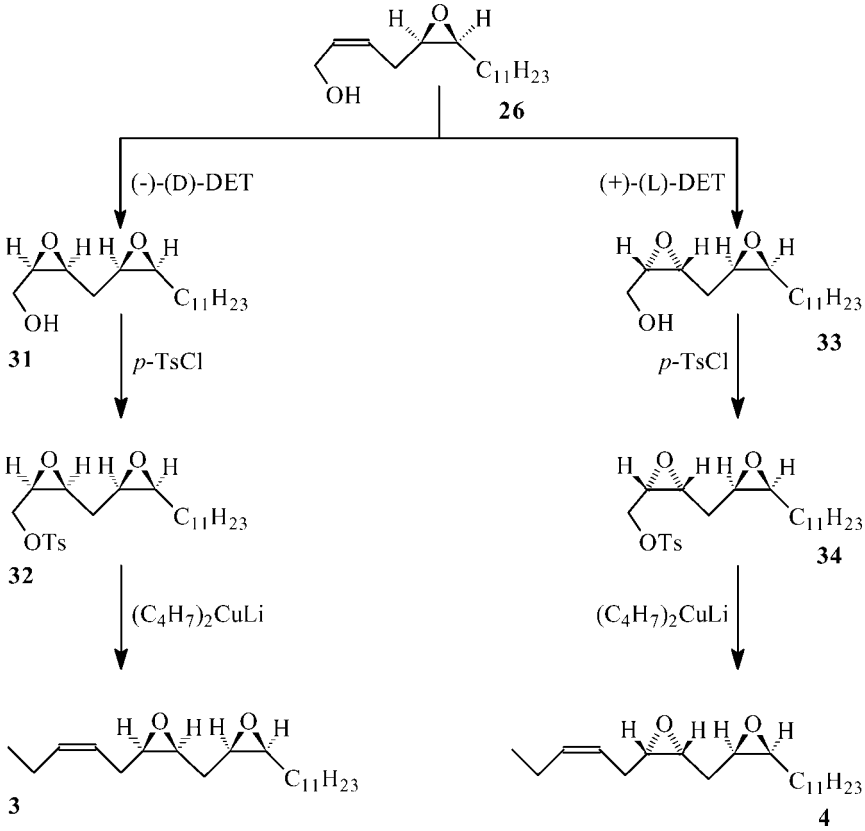


Scheme 3. Elaboration of **21** into diastereoisomers **1** and **2**.

5,6-oxirane (from D-xylose) in the precursor resulted in the expected major product and the minor product in each epoxidation being diastereoisomers, which were readily separable by medium pressure chromatography. This methodology produced the diepoxy alcohols **27**, **29**, **31**, and **33** in high diastereomeric excess.

To complete the syntheses, a butenyl group had to be attached to C-1 of each diepoxy alcohol. Compounds **27**, **29**, **31**, and **33** were tosylated to form their corresponding toluenesulfonates, **28**, **30**, **32**, and **34**, respectively. Subsequently, each diepoxy tosylate was coupled with lithium dibutenyl cuprate to yield the final products **1**–**4** (Alexakis et al., 1979; Ebata and Mori, 1989). Purification by HPLC provided enantiomeric and diastereomeric final products with >99.9% ee.

Thus, the preparation of each of the four isomers of the first discovered diepoxy moth pheromone component, (*Z*)-3-*cis*-6,7-*cis*-9,10-diepoxyhenicosene,



Scheme 4. Elaboration of **26** into diastereoisomers **3** and **4**.

in high chiral purity relied on the stereospecific formation of the *cis*-9,10-epoxy groups in compounds **20** and **24** from chirally pure D-xylose. Introduction of chiral *cis*-6,7-epoxy functionalities in compounds **27**, **29**, **31**, and **33** by asymmetric epoxidation, followed by chromatography, removed the diastereomeric impurities. Finally, copper-mediated vinyl coupling (Posner et al., 1973) of diepoxy tosylates **28**, **30**, **32**, and **34** without perturbation of the oxirane ring systems formed **1–4** from D-xylose in 0.8, 1.5, 8.1, and 7.8% overall yield.

The enantiomeric identity of the natural pheromone of the satin moth remains to be identified by field trapping or gas chromatography–electroantennogram detection (GC-EAD) studies with chirally pure materials, such as those described herein. In the initial identification of the pheromone by GC-EAD (Gries et al., 1997), the major bioactive component of the (*Z*)-3-*cis*-6,7-*cis*-9,10-diepoxyhenicosene mixture was established as the slower eluting diastereoisomer on DB 5.

Wimalaratne (1998) showed that this diastereoisomer eluted later on two other GC columns, DB23, and DB210. Yamazawa et al. (2001) have recently reported that the S^*, R^*, S^*, R^* stereoisomers (i.e., **1** or **3**) are the slower eluting diastereoisomers on DB1 and DB23, implying that these pure substances, or a mixture of these enantiomers, comprise the natural attractant. As Gries et al. (1997) have demonstrated, the S^*, R^*, S^*, R^* racemic mixture is attractive to satin moth males in the field. This racemic material can be obtained by HPLC separation of the diastereomeric mixture (Yamazawa et al., 2001). In addition, the demonstrated reduced attractancy of mixtures containing the S^*, R^*, R^*, S^* stereoisomers implies that one or both of these isomers are inhibitory so that a nonnatural blend of the diastereoisomers would not function effectively as a monitoring lure.

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EVALUATION OF THE MAJOR FEMALE *Eurytoma amygdali* SEX PHEROMONE COMPONENTS, (*Z*,*Z*)-6,9-TRICOSADIENE AND (*Z*,*Z*)-6,9-PENTACOSADIENE FOR MALE ATTRACTION IN FIELD TESTS

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Abstract—We evaluated the attraction of male almond seed wasp *Eurytoma amygdali* to the synthetic alkadienes (*Z*,*Z*)-6,9-tricosadiene and (*Z*,*Z*)-6,9-pentacosadiene and their blend in almond orchards using baited rubber septa attached to cardboard rectangular adhesive traps. The two alkadienes were recently isolated from virgin female whole body extracts and SPME collected volatiles. The alkenes (*Z*)-9-tricosene, (*Z*)-9-pentacosene, and (*Z*)-9-heptacosene, present in female extracts, were also added to the blend of the alkadienes and tested. The alkadienes tested individually attracted males when the traps were baited with doses ranging from 10 to 30 mg/trap. The maximum number of males was attracted to traps baited with 10 mg of a (*Z*,*Z*)-6,9-$C_{23:2}$:(*Z*,*Z*)-6,9-$C_{25:2}$ blend at a ratio of 7:3. Results with the three alkenes added to the blend were inconclusive because of low populations. The present study on *E. amygdali* is the first one reporting attraction of males to synthetic sex pheromone components in field trials for a Eurytomidae species. The synthetic alkadienes blend offers the potential to develop an effective system for monitoring populations of the almond seed wasp in almond orchards.

Key Words—Sex pheromone, almond seed wasp, *Eurytoma amygdali*, Hymenoptera, Eurytomidae, (*Z*,*Z*)-6,9-tricosadiene, (*Z*,*Z*)-6,9-pentacosadiene, field trials.

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INTRODUCTION

The almond seed wasp *Eurytoma amygdali* Enderlein (Hymenoptera: Eurytomidae) is an important pest of almonds in a number of European countries and the Middle East (Avidov and Harpaz, 1969; Mentzelos and Atjemis, 1970; Plaut, 1971, 1972; Talhouk, 1977; Arambourg et al., 1983; Zerova and Fursov, 1991). Almond damage can reach 90% in certain susceptible varieties. The wasp has one generation per year, and larvae overwinter inside the infested almonds that remain mummified on the almond trees. The adults emerge in spring after boring a circular hole through the pericarp. Within a few days they mate and the females start ovipositing in the unripe green almonds (Ivanov, 1960; Mentzelos and Atjemis, 1970; Plaut, 1971). The hatching larvae move through the nucellus and the embryo sac to feed on the embryo (Plaut, 1972). When the larva is fully grown, it enters diapause within the seed.

Control measures for the almond wasp are based on cultural practices such as collection and destruction of the infested almonds, and chemical control by the application of a systemic insecticide against the young larvae. Chemical treatments generally are applied 10–15 d following the peak of the adults' flight, when most of the eggs have hatched (Mentzelos and Atjemis, 1970; Talhouk, 1977). Cages with infested almonds placed in the almond orchards in early spring are used to time the application of control measures by monitoring wasp emergence. Katsoyannos et al. (1992) also used traps baited with 25 virgin females to monitor populations of the almond wasp. However, the utility of this method is limited by the high number of virgin females required per trap, their regular replacement every 4–5 d, and the trained personnel required for insect rearing and trap servicing.

Recently the two alkadienes (*Z*,*Z*)-6,9-tricosadiene [(*Z*,*Z*)-6,9-$C_{23:2}$] and (*Z*,*Z*)-6,9-pentacosadiene [(*Z*,*Z*)-6,9-$C_{25:2}$] were isolated from female whole body extracts, and volatiles were collected using the solid phase microextraction technique (SPME). The alkadienes tested individually or as a 7:3 mixture attracted males in laboratory bioassays (Krokos et al., 2001).

In this study, we tested the effectiveness of cardboard rectangular adhesive traps baited with each of the synthetic alkadienes [(*Z*,*Z*)-6,9-$C_{23:2}$, (*Z*,*Z*)-6,9-$C_{25:2}$] and their blend in trapping almond wasp males under field conditions. In 2002, different concentrations of the two alkadienes and their 7:3 blend, the ratio found in the female extracts and SPME volatiles (Krokos et al., 2001) were tested. In 2003 the alkenes (*Z*)-9-tricosene [(*Z*)-9-$C_{23:1}$], (*Z*)-9-pentacosene [(*Z*)-9-$C_{25:1}$], and (*Z*)-9-heptacosene [(*Z*)-9-$C_{27:1}$] present in female whole body extracts, were added to the alkadienes blend and tested.

The objective of these studies was the development of an effective pheromone-based trapping system for monitoring the seasonal occurrence of the almond wasp or for controlling the wasp population by mass trapping.

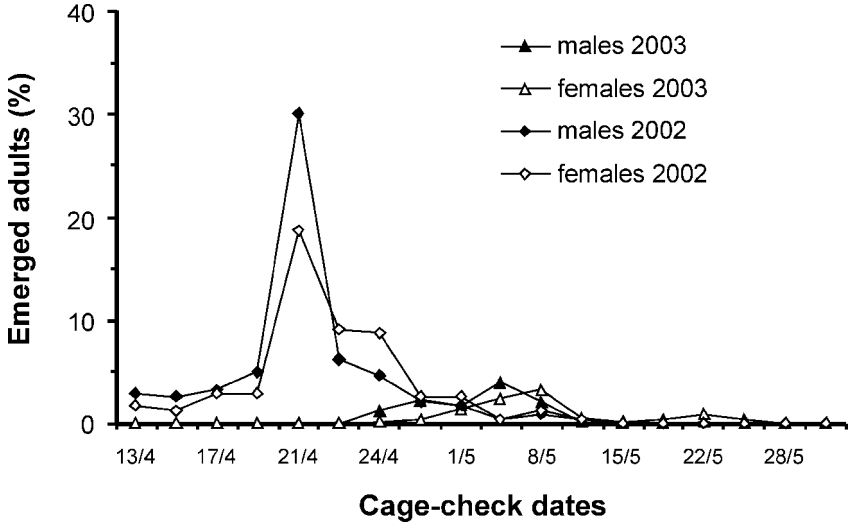

FIG. 1. Percentage of *E. amygdali* males and females emerging from infested almonds collected in early spring and placed in screen cages under field conditions in Microthivae, Magnesia during 2002 and 2003.

METHODS AND MATERIALS

Chemicals. The synthetic alkadienes and alkenes used were provided by Prof. Wittko Francke and his group (University of Hamburg, Germany). The alkadienes and the alkenes were 95–97% pure when analyzed on a 60 m $\times$ 0.25 mm $\times$ 0.1 μm film thickness DB-5 column (J & W Scientific).

Bait Dispensers. The pheromone was dispensed from rubber septa (No 100722-100EA; Aldrich Chemical Co.) loaded with hexane solutions of the test compounds. After evaporation of the solvent, the rubber septa were closed in plastic aluminum bags and stored at -20°C until use.

Traps. White cardboard rectangular adhesive traps (28 $\times$ 7 cm), with both surfaces of the trap covered with Tanglefoot (The Tanglefoot Co., Grand Rapids, MI, USA) were used. The traps were suspended vertically from trees at 1.5–2 m height, baited with rubber septa loaded with various doses of the two alkadienes (Z,Z)-6,9-$C_{23:2}$ and (Z,Z)-6,9-$C_{25:2}$, a 7:3 binary blend, or mixtures of the binary blend and each of the alkenes (Z)-9-$C_{23:1}$, (Z)-9- $C_{25:1}$, and (Z)-9-$C_{27:1}$. The rubber septa were placed in a hole in the center of the sticky trap in order to have sufficient pheromone emission from both sides.

Field Trapping Sites. In 2002, trapping experiments were conducted around Mikrothivae (Magnesia, Central Greece), one of the main almond-producing

areas of Greece. The almond orchards were approx. 9 ha, with trees >25 years old (var. Texas). The orchards were conventionally cultivated and 1–2 chemical treatments were applied annually against the almond wasp.

In 2003, the trapping experiments were carried out in the same orchards and two abandoned orchards. The first was located in the area of Latomia, Magnesia near Volos, 25 km from the Microthivae area. The second was a medium size orchard with ca 150 almond trees abandoned for many years in Ag. Paraskevi, Attikis near Athens.

Traps were deployed in a block design with one replicate of each treatment incorporated into each block. Traps were placed every five trees so that individual treatments were >40 m apart, and the blocks were sited >70 m apart. After each sampling date, the traps were rotated to the next trap location within each block, and captured males were counted and removed from the traps.

Statistical Analysis. The number of captured males was log transformed [log $(x + 1)$], then submitted to one-way ANOVA using the General Linear Models of SAS (SAS Institute, 1995). Means were compared by the Tukey–Kramer (HSD) test at $P = 0.05$ (Sokal and Rohlf, 1995). Finally, to examine the synchronization between trap catches and male emergence from the cages, the correlation coefficients of trap–cage pairs were examined, separately for each year. The coefficients were tested for difference from zero by using the two-tailed t test, at $P = 0.01$ (Snedecor and Cochran, 1989).

Field Experiments

Test 1. Microthivae, Magnesia: 11 April–15 May 2002. Five doses (1, 5, 10, 20, and 30 mg) of the (Z,Z)-6,9-$C_{23:2}$ and (Z,Z)-6,9-$C_{25:2}$ alkadienes and their blend (7:3) were evaluated. Traps were set up in three blocks (replicates) and inspected every 2 d, for a total of 17 trap-check dates, providing 51 data points (17 inspections × 3 replicates).

Test 2. Microthivae, Magnesia: 23 April–23 May 2003. Traps were baited with rubber septa loaded with 3 mg of (Z,Z)-6,9-$C_{23:2}$, the binary blend (2.3 mg of (Z,Z)-6,9-$C_{23:2}$ and 0.7 mg of (Z,Z)-6,9-$C_{25:2}$), and mixtures of the binary blend (2.3 mg (Z,Z)-6,9-$C_{23:2}$: 0.7 mg (Z,Z)-6,9-$C_{25:2}$) and 0.7 mg of each one of the alkenes, (Z)-9-$C_{23:1}$, (Z)-9-$C_{25:1}$, and (Z)-9-$C_{27:1}$. The same experimental design was kept, for a total of 15 check dates providing 45 data points.

Test 3. Latomia, Volos Magnesia: 24 April–18 May 2003. Traps were baited with rubber septa loaded either with 10 mg of (Z,Z)-6,9-$C_{23:2}$ or the 7:3 blend of the two alkadienes [(Z)-9-$C_{23:2}$ and (Z)-9-$C_{25:2}$] using doses of 1, 3, 5, and 10 mg. Traps were inspected 8 times every 3 d, for a total of 24 data points.

Test 4. Agia Paraskevi, Attikis: 22 April–12 May 2003. Traps were baited with rubber septa loaded with 5 mg of (Z,Z)-6,9-$C_{23:2}$, the 7:3 binary blend of

(*Z*,*Z*)-6,9-$C_{23:2}$ and (*Z*,*Z*)-6,9-$C_{25:2}$, and mixtures of the binary blend and each one of the alkenes (*Z*)-9-$C_{23:1}$, (*Z*)-9-$C_{25:1,}$ and (*Z*)-9-$C_{27:1.}$ Traps were inspected 7 times every 3 d for a total of 21 data points.

Emergence Rate of Almond Wasp. The emergence rate of the almond wasp was monitored during 2002 and 2003. In the Microthivae experimental orchard, three cages, one on each block, containing 300 infested almonds were placed on a bench of 40 cm height. The infested almonds were collected prior to trap placements from the same area. On each trap check date, the number of emerged wasps (males and females) were recorded and removed from the cage.

RESULTS AND DISCUSSION

Preliminary wind tunnel bioassays showed that the synthetic alkadienes elicited strong responses from males, with the maximum response observed when the blend of the two alkadienes was tested (Krokos et al. 2001, and personal observations).

The first field test during 2002 confirmed the wind tunnel observations (Table 1). Traps baited with each of the alkadienes attracted males and attraction increased with dose. Catches at the doses of 10, 20, or 30 mg attracted similar numbers of males for each of the components. For these three doses, mean trap catches were greater than catches in the traps baited with 1, 3, and 5 mg (Table 1).

Catches in traps baited with 1–10 mg of the binary blend of [(*Z*,*Z*)-6,9-$C_{23:2}$:(*Z*,*Z*)-6,9-$C_{25:2}$] at a ratio of 7:3 were greater than catches in traps baited with similar doses of each one of the two alkadienes (Table 1). However, male catches in traps baited with 20 and 30 mg of the blend were not different from catches in traps baited with equal doses of each of the two alkadienes.

All doses of the blend attracted males, indicating that both components are essential for maximum male attraction under field conditions. Contrary to the results obtained with traps baited with the individual components, doses of 20 or 30 mg (13.33 and 6.66 or 20 and 10 mg of [(*Z*,*Z*)-6,9-$C_{23:2}$ and (*Z*,*Z*)-6,9-$C_{25:2}$] of the binary blend attracted fewer males than traps baited with 10 mg (6.66 of (*Z*,*Z*)-6,9-$C_{23:2}$ and 3.33 of (*Z*,*Z*)-6,9-$C_{25:2}$3), suggesting that larger doses of the blend may be inhibitory. Inhibitory effects of high pheromone doses and release rates have been reported for other insect species (Roelofs and Cardé, 1974; Cardé and Baker, 1984; Grant et al., 1989; Knutson et al., 1998).

Similar results were obtained in 2003 where four concentrations of the binary blend were tested. The traps baited with 10 mg attracted significantly more males that the other concentrations tested (Table 2). Traps baited with 10 virgin females (2- to 4-d-old) placed in small screen cages attracted fewer males than traps baited with 10 mg of the binary blend (Table 2). The results show that traps baited with 10 mg of the binary blend is most effective for monitoring *E. amygdali* populations.

TABLE 1. MEAN NUMBER OF MALE *E. amygdali* CAPTURED PER TRAP COUNT IN CARDBOARD STICKY TRAPS BAITED WITH VARIOUS DOSES OF THE TWO ALKADIENES (Z,Z)-6,9-$C_{23:2}$ AND (Z,Z)-6,9-$C_{25:2}$, AND VARIOUS DOSES OF THEIR BLEND AT A RATIO OF 7:3. MICROTHIVAE, MAGNESIA 11 APRIL–15 MAY 2002

Dose (mg)	Control Mean (SE)	(Z,Z)-6,9-$C_{23:2}$ Mean (SE)	(Z,Z)-6,9-$C_{25:2}$ Mean (SE)	(Z,Z)-6,9-$C_{23:2}$ + (Z,Z)-6,9-$C_{25:2}$ Mean (SE)	ANOVA statistics
—	0.0	—	—	—	—
1	—	0.0 (0.0)Db	0.0 (0.0)Cb	0.7 (0.14)Ba	$F = 21.07$, $P < 0.001$
3	—	0.1 (0.02)CDb	0.0 (0.0)Cb	0.9 (0.17)Ba	$F = 16.71$, $P < 0.001$
5	—	0.3 (0.09)CDb	0.2 (0.09)BCb	1.4 (0.37)Ba	$F = 11.16$, $P < 0.001$
10	—	0.6 (0.13)BCb	0.4 (0.11)ABb	2.1 (0.56)Aa	$F = 18.23$, $P < 0.001$
20	—	0.9 (0.21)ABa	0.5 (0.13)ABa	0.7 (0.14)Ba	$F = 0.82$, $P = 0.438$
30	—	1.1 (0.31)Aa	0.7 (0.11)Aa	0.9 (0.26)Ba	$F = 1.32$, $P = 0.268$
ANOVA statistics	—	$F = 11.16$ $P < 0.001$	$F = 4.93$ $P < 0.001$	$F = 12.91$ $P < 0.001$	

Note. Means followed by the same small case letter within each row are not significantly different (in all cases df = 2, 150); means followed by the same capital letter within each column are not significantly different (in all cases df = 5, 300); Tukey–Kramer HSD test at $P = 0.05$.

TABLE 2. MEAN NUMBER OF MALE *E. amygdali* CAPTURED PER TRAP COUNT IN CARDBOARD STICKY TRAPS BAITED WITH 10 MG OF (Z,Z)-6,9-$C_{23:2}$ AND FOUR DOSES OF THE TWO ALKADIENES (Z,Z)-6,9-$C_{23:2}$ AND (Z,Z)-6,9-$C_{25:2}$ BLEND AT A RATIO 7:3. LATOMIA, VOLOS, MAGNESIA, 23 APRIL–15 MAY, 2003

Dose (mg)	10 Females Mean (SE)	(Z,Z)-6,9-$C_{23:2}$ Mean (SE)	Z, (Z, Z)-6,9-$C_{23:2}$ + (Z,Z)-6,9-$C_{25:2}$ Mean (SE)
—	0.07 (0.04)b	—	—
1	—	0.0b	0.02 (0.02)b
3	—	0.0b	0.05 (0.03)b
5	—	0.0b	0.02 (0.02)b
10	—	0.18 (0.07)b	0.52 (0.11)a

Note. Means followed by the same letter are not significantly different ($F = 12.35$, df $= 4, 220$, $P < 0.001$; Tukey–Kramer HSD test at $P = 0.05$).

In the other two field tests during 2003, at Microthivae and Aghia Paraskevi areas, where 3 and 5 mg, respectively, of the (Z,Z)-6,9-$C_{23:2}$, the binary blend and mixtures of the binary blend and each of the three alkenes (Z)-9-$C_{23:1}$, (Z)-9-$C_{25:1}$, and (Z)-9-$C_{27:1}$ were tested, results were inconclusive. Few males (4 and 8 in Microthivae, 9 and 18 in Aghia Paraskevi) were caught in traps baited with the (Z,Z)-6,9-$C_{23:2}$ and the binary blend, respectively. Traps baited with mixtures where each one of the three alkenes was added to the binary blend attracted no males. Low trap catches might be attributed to the dose of pheromone used and the low wasp emergence in this year (Figure 1).

The seasonal flight of *E. amygdali* was monitored at Microthivae with traps baited with 10 mg of the binary blend in both 2002 and 2003. In 2002, the flight started on April 13, and wasp population peaked from April 19 to April 30 (Figure 2A). In 2003, the flight started on April 24, and the wasp population peaked from May 1 to May 8 (Figure 2B). According to field cage observations from previous years the flight activity of *E. amygdali* in the area of Microthivae starts the first half of April.

In 2002, flight activity recorded with the pheromone traps compared well with previous findings, but in 2003 the flight activity was delayed approximately 2 weeks and the numbers of males caught in the pheromone traps were considerably lower because of unfavorable weather conditions.

Male catches in pheromone traps baited with 10 mg of the binary blend were correlated with the number of males emerging from infested almonds by using the cage method traditionally used by the farmers to detect wasp flight activity. The correlation coefficients of trap catches in relation to wasp emergence were $r = 0.691$, $N = 48$ for 2002 and $r = 0.731$, $N = 44$ for 2003 (both different from 0 at $P < 0.01$).

Wasp emergence was significantly lower in 2003 with 40% of the infested almonds producing wasps compared to 90% in 2002 (Figure 1). Inspection of

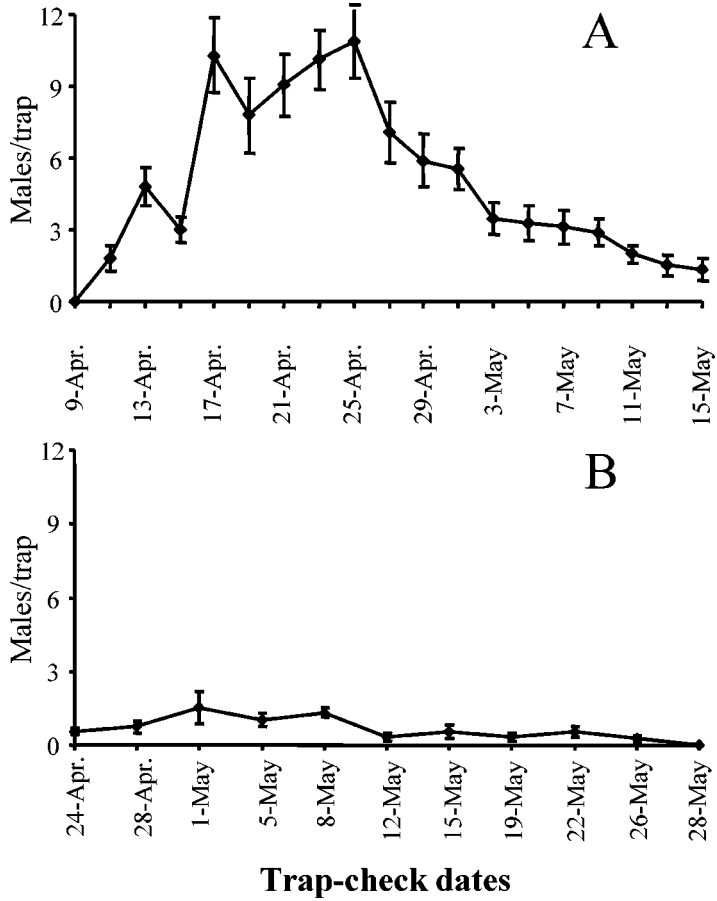

FIG. 2. Flight activity of *Eurytoma amygdali* males evaluated with pheromone traps baited with 10 mg of the two alkadienes (*Z*,*Z*)-6,9-$C_{23:2}$ and (*Z*,*Z*)-6,9-$C_{25:2}$ at a ratio of 7:3, in Microthivae, Magnesia during 2002 (A) and 2003 (B).

infested almonds in 2003 revealed that a high percentage of the larvae were still in diapause, indicating that low temperatures in early spring were unfavorable to terminate diapause. According to Tzanakakis et al. (1991), *E. amygdali* diapausing larvae require certain thermal optima and threshold to terminate diapause. If these optima are not reached the larvae remain in a prolonged diapause and complete their life cycle in 2 years or more.

The Eurytomidae family includes species that reproduce parthenogenetically, species in which males are rare, and species that reproduce sexually (Nadel and Peña, 1991). The response of Eurytomidae to chemical cues has been reported

for the clover seed wasp *Bruchophagous gibbus* and the alfalfa seed wasp *Bruchophagus roddi* (Kamm and Buttery, 1986; Light et al., 1992). Volatiles released by their host plants, red clover and alfalfa, play a key role in host selection by females. Based on electrophysiological and behavioral studies and field tests with virgin females as baits, Leal et al. (1997) reported the occurrence of a long-range female-released sex pheromone in a sexually reproducing species, *Bephratelloides pomorum*.

The present study on *E. amygdali* is the first to report male attraction in field trials to traps baited with the female-produced sex pheromone components for a Eurytomidae species.

Long chain mono- or poly-unsaturated hydrocarbons have been reported as major or secondary sex pheromone components for many insect species. For example, female sex pheromones in arctiid moths (Lepidoptera: Arctiidae) are mainly polyunsaturated hydrocarbons and the related epoxides (Descoins et al., 1989). In the Diptera, long chain hydrocarbons eliciting male courtship behavior have been reported for many species such as the house fly *Musca domestica* (Carlson et al., 1971) and several *Drosophila* species (Antony and Jallon, 1982; Antony et al., 1985; Oguma et al., 1992; Nemoto et al., 1994).

Results obtained from our field trapping experiments during the 2 years suggest that cardboard rectangular adhesive traps baited with 10 mg of the binary blend of (Z,Z)-6,9-$C_{23:2}$ and (Z,Z)-6,9-$C_{25:2}$ can be used for monitoring *E. amygdali* populations, and timing application of control measures. Further experimentation is required for the optimization of various parameters affecting male attraction, such as the importance of the stereomeric purity and the isomerization of the (Z,Z)-alkadienes to other isomers. The type and shape of the trapping devices also should be considered for the continued development of an efficient pheromone-based method for monitoring and control of this pest.

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CHEMICAL AND PHYSICAL SIGNALS MEDIATING CONSPECIFIC AND HETEROSPECIFIC AGGREGATION BEHAVIOR OF FIRST INSTAR STINK BUGS

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Abstract—We investigated cues that mediate the aggregation behavior of immature pentatomid bugs by using nymphs of six different pentatomid bug species (*Nezara viridula, Acrosternum hilare, Chlorochroa ligata, Chlorochroa sayi, Thyanta pallidovirens*, and *Euschistus conspersus*). When first instars of any two species were put together in a Petri dish, they readily formed heterospecific aggregations similar to their natural conspecific aggregations. The chemical profiles of first and second instar nymphs of each species were determined by solvent extraction with pentane, followed by GC–MS analysis. Immature bugs of the different species had some compounds in common, and some that were more species specific. Within a species, there were distinct differences in the profiles of compounds extracted from first and second instars. Bugs did not aggregate around untreated polysulfone beads (1 mm diam) that were glued together in groups approximating bug egg masses, suggesting that tactile cues alone were insufficient to induce aggregation. Furthermore, when tested over a range of doses, groups of polysulfone beads treated with crude whole-body extracts of bugs did not induce or maintain aggregations. However, first instar *N. viridula* nymphs did respond to beads treated with two of the three major components of bug extracts. 4-Oxo-(*E*)-2-decenal induced significant aggregations at two doses, whereas tridecane, the major component in extracts from all six species, did not, and (*E*)-2-decenal was repellent. The repellence of (*E*)-2-decenal may explain why we and previous researchers were unable to induce aggregations of first instar *N. viridula* using whole-body extracts.

Key Words—aggregation pheromone, allomone, Pentatomidae, *Acrosternum hilare, Chlorochroa sayi, Chlorochroa uhleri, Euschistus conspersus, Nezara viridula, Thyanta pallidovirens*, heterospecific aggregation, 4-oxo-(*E*)-2-decenal, tridecane, (*E*)-2-decenal.

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INTRODUCTION

The Pentatomidae form one of the largest families within the true bugs (Heteroptera), comprising about 4100 of the estimated 36,100 described species of Heteroptera (Panizzi et al., 2000). Pentatomid bugs are commonly known as "stink bugs" because in response to disturbance or aggression, immatures and adults produce irritating defensive secretions with a disagreeable odor from large dorsal abdominal glands (DAGs) or metathoracic glands (MTGs) (Aldrich, 1988). However, components of these secretions may also function as pheromones for immature bugs. When released in smaller quantities and/or different ratios, it has been suggested that components of the secretions can act as aggregation pheromones or arrestants, inducing first instar nymphs to cluster (Ishiwatari, 1976; Lockwood and Story, 1985; Borges and Aldrich, 1992; Pavis et al., 1994). Several benefits of this aggregative behavior have been proposed for immature bugs, including protection against desiccation, accelerated development of aggregated bugs versus single bugs, reduced mortality, lower predation rates, and better adherence to substrates to minimize dislodging by wind or rain (Kiritani, 1964; Lockwood and Story, 1986). The tendency to aggregate is quite strong: if clusters of nymphs are manually dispersed, within a few minutes they begin to reaggregate, with stable clusters formed again within 1 hr (Ishiwatari, 1976).

It has been proposed that the formation and maintenance of aggregations of stink bug nymphs is mediated by several types of stimuli, including tactile, visual, and olfactory signals, but the results from several studies are not entirely congruent (Lockwood and Story, 1986; Borges and Aldrich, 1992; Pavis et al., 1994). For example, Ishiwatari (1976) presented data suggesting that first instar *Eurydema rugosa* aggregate in response to (*E*)-2-hexenal in combination with tactile cues (cork fragments glued together in clusters to mimic egg masses). Aggregations formed equally well under light or dark conditions, suggesting that visual cues were not important. Aggregative behavior was eliminated in antennectomized individuals, providing support for a role for olfactory stimuli in the formation of aggregations. Ishiwatari (1976) also made the interesting observation that two closely related species readily formed heterospecific aggregations, whereas two more distantly related species did not. Lockwood and Story (1985) carried out methodical studies with *Nezara viridula* immatures, and found that newly hatched individuals, which contained no detectable chemical signals, aggregated in response to tactile cues from the egg shells. However, as the hatchlings aged, they began to produce chemical signals, and after several days, the bugs moved off the eggshells and formed new aggregations, apparently in response to chemical cues. Lockwood and Story (1985) initially identified tridecane as the compound mediating the formation of *N. viridula* aggregations, but they failed to find and test 4-oxo-(*E*)-2-decenal, another major constituent of the nymphal extracts. A subsequent study by Borges and Aldrich (1992) identified 4-oxo-(*E*)-2-decenal from extracts of first instar

N. viridula, *Thyanta perditor*, *Euschistus heros*, and *Acrosternum aseadum*, and showed that it was produced only during the first instar. Although these authors suggested that 4-oxo-(*E*)-2-decenal may function as an aggregation pheromone, they were unable to induce the formation of *N. viridula* aggregations using whole body extracts containing 4-oxo-(*E*)-2-decenal plus other chemicals. In a follow-up study, Pavis et al. (1994) confirmed that 4-oxo-(*E*)-2-decenal was produced only by first instar *N. viridula*, and presented data indicating that this compound functions as an attractant and an arrestant for first instars. Somewhat surprisingly, second instars were also attracted and arrested, even though they did not produce it.

Our goal was to examine the aggregative behavior of first instar nymphs of a number of stink bug species, specifically, the phytophagous pentatomid species *Acrosternum hilare* (Say), *Chlorochroa ligata* (Say), *Chlorochroa sayi* Stal, *Euschistus conspersus* Uhler, *Nezara viridula* (L.), and *Thyanta pallidovirens* Stal. All six of these species are important agricultural pests, and they are all quite closely related, all being in the tribe Pentatomini.

Our specific objectives were (1) to compare the chemical profiles of first and second instar nymphs of each species by coupled gas chromatography–mass spectrometry (GC–MS) analysis of whole body extracts of nymphs; (2) to determine whether aggregations of first instar nymphs, and potentially the cues mediating aggregative behavior, were species specific; (3) to determine whether the extracts from first instar nymphs, or components of the extracts, mediated the formation of aggregations, using *N. viridula* as the test species; (4) to determine whether tactile cues also were important in formation of aggregations by first instar nymphs.

METHODS AND MATERIALS

Insects. All six species were reared in identical fashion at the University of California, Riverside, in an environmental chamber at $26 \pm 1^{\circ}$C, 55% RH, and with a photoperiod of 16:8 hr (L:D) provided with fluorescent lights. Both nymphs and adults were fed with organically grown green beans, raw peanuts, and sunflower seeds. Adults also were fed with bouquets of alfalfa and/or seasonal weeds in a jar of water. Food was changed three times per week. Adults were maintained in a wooden cage ($76 \times 43 \times 43$ cm) with a glass top. Egg masses were collected daily from paper towels placed on the cage bottom and on top of the alfalfa bouquet, and placed in 15 cm diam plastic Petri dishes. After hatching, the first instars were held in the dishes until they moulted, then reared in circular plastic containers (20×15 cm, with two 4 cm circular holes on opposite sides of each container covered with brass screening for ventilation) until the final moult.

Preparation and Analysis of Extracts of First and Second Instars. Groups of nymphs of known age were isolated in a 7 cm plastic Petri dish the day before being extracted. Nymphs were killed by freezing, then transferred to a 2 ml vial (20–40 nymphs/extract), and immersed for 15 min in a quantity of pentane just sufficient

to submerge them. The extracts were transferred to clean vials, and as required, concentrated by leaving the open vials in a fumehood for a few minute before analysis. Extracts were analyzed by GC (Hewlett-Packard 5890 Series II; Hewlett-Packard, Santa Clarita CA; H-P) in splitless mode on a DB-5 column (20 m $\times$ 0.32 mm ID; J&W Scientific, Folsom CA), using a program of 50°C/min, 10°C/min to 250°C. Compounds were tentatively identified by interpretation of spectra and/or matchup with literature or NIH-NBS computer database spectra. Identifications were confirmed by matchup of spectra and retention times with those of authentic standards (see below). To estimate the amount of the major volatiles produced by nymphs, three replicates of 40 *N. viridula* first instar nymphs were extracted with 100 μl of pentane for 15 min, an internal standard was added (20 ng methyl phenylacetate in 20 μl CH_2Cl_2), and 1 μl of the extract was injected in splitless mode on an H-P 5890 GC equipped with a DB-1 column (30 m $\times$ 0.32 mm ID, 3 μm film), with flame ionization detection. Injector and detector temperatures were 250° and 275°C, respectively, with a constant carrier gas velocity of 35 cm/sec helium. The oven temperature was held at 35°C for 1 min, then programmed at 5°C/min to 250°C, where it was maintained for 20 min. Data were collected with an H-P 3396 integrator and the detected volatiles were quantified based on comparison of the integrated peak areas of tridecane, 4-oxo-(*E*)-2-decenal and (*E*)-2-decenal with that of the internal standard.

Formation of Heterospecific Aggregations. Groups of approximately 40 first instar nymphs (24–48-hr-old) of each of two species were introduced into 4 cm diam Petri dishes with filter paper discs in the bottom, using a small damp paintbrush to avoid injuring the nymphs. The dishes were held under ambient laboratory light and temperature (22 $\pm$ 2°C), and examined 15 min, 30 min, 1 hr, 2 hr, and 5 hr after adding the nymphs. At each observation, the number of aggregations and the numbers of bugs of each species in each aggregation were recorded. All possible combinations of two species were tried, resulting in a total of 15 combinations from the six study species. The data were analyzed using Fisher 2 $\times$ 2 tables (Statistica 5.1, Statsoft Italia, 1997).

Bioassay of Tactile Cues, and Combinations of Tactile Cues with Chemical Cues. To determine whether tactile cues might be involved in the aggregation response, model aggregations were made by gluing polysulfone beads (1 mm diam; Bend Research, Inc. Bend, OR) together with a 5% aqueous solution of agar, in groups simulating pentatomid egg masses (7–9 beads per mass, two masses per 4 cm filter paper disc). The filter papers were placed in the bottoms of plastic Petri dishes as described above, and one mass was treated with 10 μl of a crude pentane extract of *N. viridula* first instar nymphs, with the other treated with an equal volume of clean solvent as a control. In assays testing synthetic compounds, dummy egg masses were treated with pentane solutions of test compounds (doses listed in Table 5). Assays with untreated dummy egg masses were also done to test the effects of tactile cues alone on bug aggregation behavior. Each assay was

replicated ×5 with 10 first instar *N. viridula* nymphs, with the numbers of bugs forming aggregations on each dummy egg mass being counted at 15 min, 30 min, 1 hr, 2 hr, and 5 hr after introduction of the nymphs. The data were analyzed using Wilcoxon matched-pairs tests (Statistica 5.1, Statsoft Italia, 1997).

Chemicals. 4-Oxo-(*E*)-2-hexenal and 4-oxo-(*E*)-2-octenal were synthesized as previously described (Marques et al., 2000). 4-Oxo-(*E*)-2-decenal was prepared by the same route, substituting heptanal in the first step of the synthetic sequence instead of propanal and pentanal, respectively. All other compounds were obtained from Aldrich Chemical Co. (Milwaukee, WI).

RESULTS

Chemical Profiles of First and Second Instar Nymphs. The chemical profiles of first and second instar nymphs of each species are listed in Tables 1 and 2, respectively, and a comparison between the first and second instars of each species, over all species, is shown in Figure 1. On average, the amounts of compounds produced by *N. viridula* first instar nymphs, for two of the most abundant compounds, were 12.5 ± 2.9 ng of tridecane and 23.4 ± 7.7 ng of 4-oxo-(*E*)-2-decenal.

Extracts of the first instars of all species contained tridecane as a major component (28.8–64.9%), variable amounts of (*E*)-2-decenal (0.3–10.4%), and small amounts of dodecane, pentadecane, and tridecene. The two *Chlorochroa* species had no detectable 4-oxo-(*E*)-2-decenal, whereas this compound was a major component (9.01–56.7%) of the extracts of the other four species. Conversely, extracts of both *Chlorochroa* spp. contained large amounts of (*E*)-2-octenal as a major component (15.8 and 29.2%, respectively), a compound that was absent from extracts of the other four species. Small amounts of 4-oxo-(*E*)-2-hexenal, 4-oxo-(*E*)-2-octenal, and 4-oxo-(*E*)-2-nonenal (tentative identification only) also were identified in several extracts. Tetradecanal was found only in *Euschistus conspersus* extracts, in which it formed 13.6% of the total. Other minor components found in the various extracts are listed in Tables 1 and 2.

Extracts of the second instar nymphs bore many similarities to those from the first instars (Figure 1), with tridecane being a major component in the extracts of all species. However, 4-oxo-(*E*)-2-decenal was detected in only trace amounts from two of the four species in which it had been a major component in the extracts from first instars, with concomitant increases in the amount of (*E*)-2-decenal in the extracts from second instars. Extracts of second instars of all species also contained moderate amounts of 4-oxo-(*E*)-2-hexenal, and small to moderate amounts of (*E*)-2-hexenal, dodecane, tridecene, and pentadecane (Table 2). Tetradecanal was a major component (28.3%) of *E. conspersus* extracts, but only present as a trace component in extracts from four of the other five species (Table 2).

TABLE 1. RELATIVE PERCENTAGES OF VOLATILE COMPOUNDS IN WHOLE BODY EXTRACTS OF FIRST INSTAR NYMPHS OF SIX PENTATOMID BUG SPECIES[a]

Compound	Species					
	N. viridula	*A. hilare*	*T. pallidovirens*	*E. conspersus*	*C. ligata*	*C. sayi*
(*E*)-2-Hexenal	—	—	—	—	—	0.28
4-Oxo-(*E*)-2-hexenal	1.78	—	1.02	3.39	0.36	2.42
Unconjugated octenal	—	—	—	—	2.1	3.35
M/*z* 112 (isomer of 4-oxo-(*E*)-2-hexenal)	1.69	—	—	—	—	0.69
(*Z*)-2-Octenal	—	—	—	—	0.63	1.25
(*E*)-2-Octenal	—	—	—	—	15.84	29.19
(*E*)-2-Octenol	—	—	—	—	—	0.49
Undecane	—	0.05	0.09	—	—	0.11
Linalool	—	—	—	2.28	—	—
4-Oxo-(*E*)-2-octenal	—	—	—	0.65	—	—
Unconjugated decenal[b]	—	0.20	0.32	0.05	—	—
Dodecane	0.56	1.36	1.82	0.7	1.22	1.23
(*Z*)-2-Decenal[b]	0.21	0.32	0.37	0.2	—	—
(*E*)-2-Decenal	2.22	10.34	10.41	3.37	0.3	1.46
Tridecene[b]	0.20	0.24	0.21	0.22	0.25	0.24
Tridecane	28.84	38.65	36.33	37.39	64.94	45.20
4-Oxo-(*E*)-2-decenal	56.70	37.37	9.01	21.87	—	—
Tetradecane	—	0.04	—	0.17	—	—
Pentadecane	0.11	0.14	0.18	0.21	0.14	0.10
Tetradecanal	—	—	—	13.55	—	—
Unknown	—	0.21	—	0.37	—	—

[a] Groups of 20–40 bugs from the same cohort were used to prepare each extract.
[b] Identifications are tentative only based on interpretation of mass spectra, and retention times relative to related compounds and isomers of known structure.

Formation of Conspecific and Heterospecific Aggregations. All possible combinations of two species readily formed heterospecific aggregations (Figure 2A, Tables 3 and 4), indicating that at least some of the cues mediating aggregation formation were common to all six of the study species. Small heterospecific aggregations (~3–4 bugs) began to form within 15 min of the bugs being put together in a Petri dish. After 5 hr, the overall numbers of heterospecific aggregations were similar, but the number of bugs in each aggregation had increased to around 15–20 bugs (Table 3). Surprisingly, the numbers of bugs in heterospecific aggregations were larger than the numbers in conspecific aggregations ($P < 0.05$) in all but one of the 15 species pairs tested. Only in the case of the *A. hilare* and *C. sayi* pairs were the numbers of nymphs in heterospecific and conspecific aggregations approximately the same ($P > 0.05$) (Table 4).

Chemical Cues Mediating Formation of Aggregations. When introduced into arenas containing two groups of untreated beads (Figure 2B), aggregations of

TABLE 2. RELATIVE PERCENTAGES OF VOLATILE COMPOUNDS IN WHOLE BODY EXTRACTS OF SECOND INSTAR NYMPHS OF SIX PENTATOMID BUG SPECIES[a]

Compound	Species					
	N. viridula	*A. hilare*	*T. pallidovirens*	*E. conspersus*	*C. ligata*	*C. sayi*
(*E*)-2-Hexenal	7.29	0.32	0.32	0.28	1.19	2.70
4-Oxo-(*E*)-2-hexenal	3.67	2.20	2.94	9.30	6.70	2.71
Unconjugated octenal	—	—	—	—	—	0.45
m/*z* 112 (isomer of 4-Oxo-(*E*)-2-hexenal)	0.19	—	0.99	0.47	1.58	0.46
(*Z*)-2-Octenal	—	—	—	0.47	—	0.27
(*E*)-2-Octenal	1.08	1.24	0.25	—	0.64	15.44
(*E*)-2-Octenol	—	—	—	—	—	0.77
Undecane	0.05	0.06	0.10	—	—	0.77
Linalool	—	—	—	4.15	—	—
4-Oxo-(*E*)-2-octenal	—	—	—	0.35	—	—
Unconjugated decenal[b]	0.44	0.55	0.38	—	0.67	—
Dodecane	1.69	1.93	1.88	0.67	2.2	0.75
(*Z*)-2-Decenal[b]	0.29	0.39	0.28	—	0.48	0.10
(*E*)-2-Decenal	27.82	23.47	19.09	—	29.20	0.10
Tridecene[b]	0.25	0.16	0.28	0.28	0.26	0.18
Tridecane	34.35	29.07	37.42	49.26	44.19	26.59
4-Oxo-(*E*)-2-decenal	—	0.09	0.08	—	—	—
Tetradecane	0.04	—	0.05	0.28	—	0.01
Pentadecane	0.19	0.13	0.27	0.22	0.16	0.07
Tetradecanal	0.05	0.04	—	28.27	0.08	0.02
Unknown	0.24	0.11	0.20	0.53	0.28	0.13

[a] Groups of 20–40 bugs from the same cohort were used to prepare each extract.
[b] Identifications are tentative only based on interpretation of mass spectra and retention times relative to related compounds and isomers of known structure.

N. viridula first instar nymphs occurred randomly and never on the beads ($P < 0.005$), showing that the tactile stimulus of the untreated polysulfone beads did not induce aggregation behavior. When tested with first instar *N. viridula*, the crude extract of nymphs, either neat, or at 2-, 10-, or 100-fold dilutions, also was indistinguishable from the solvent controls in inducing aggregation ($P > 0.05$). Tridecane, a major component in extracts from all six species, did not induce aggregations at any of the four doses tested (1000 μg, 100 μg, 10 μg, and 1 μg) ($P > 0.05$) (Table 5).

The two lowest doses of 4-oxo-(*E*)-2-decenal (5 μg and 0.5 μg) induced more nymphal aggregation on the treated egg masses than on the controls ($P < 0.01$), whereas responses to the higher doses (50 μg and 25 μg) were not significantly different from those to the controls ($P > 0.05$). In contrast, (*E*)-2-decenal was repellent at all doses tested (1000 μg, 100 μg, 10 μg, 1 μg) ($P < 0.01$ for all doses) (Table 5).

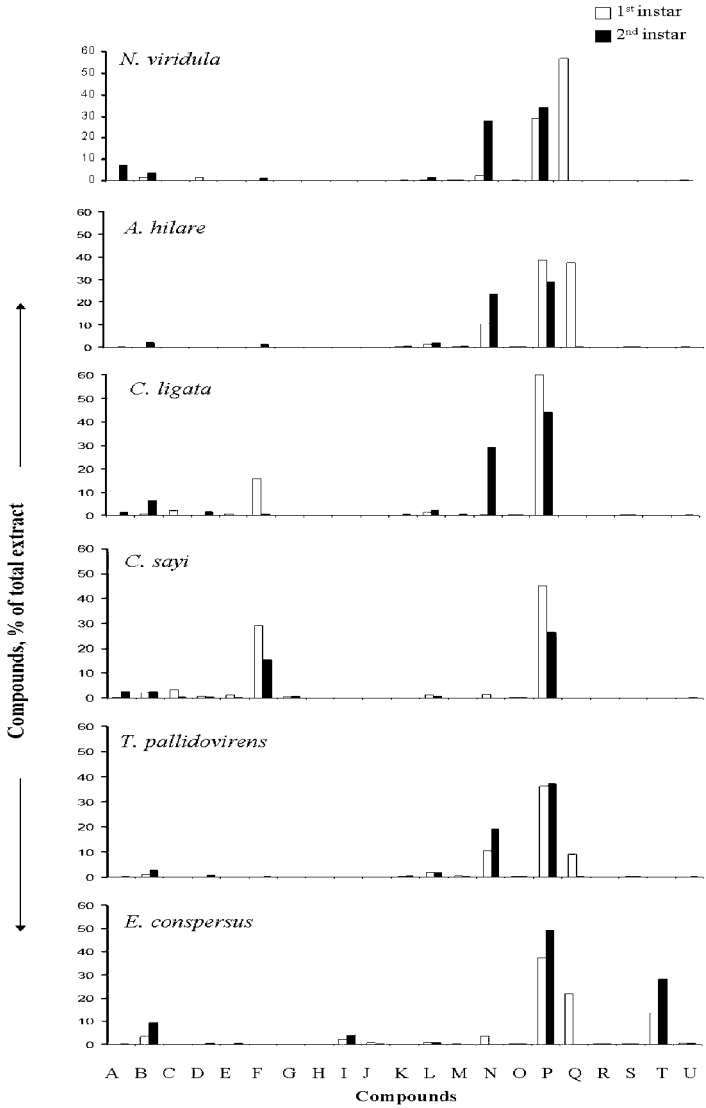

FIG. 1. Relative percentages of volatile compounds in extracts from first and second instar nymphs of six species of pentatomid bugs. A = (*E*)-2-hexenal, B = 4-oxo–(*E*)-2-hexenal, C = unconjugated octenal, D = *m*/*z* 112 (isomer of 4-oxo–(*E*)-2-hexenal), E = (*Z*)-2-octenal, F = (*E*)-2-octenal, G = (*E*)-2-octenol, H = undecane, I = linalool, J = 4-oxo-(*E*)-2-octenal, K = unconjugated decenal, L = dodecane, M = (*Z*)-2-decenal, N = (*E*)-2-decenal, O = tridecene, P = tridecane, Q = 4-oxo-(*E*)-2-decenal, R = tetradecane, S = pentadecane, T = tetradecanal, U = unknown.

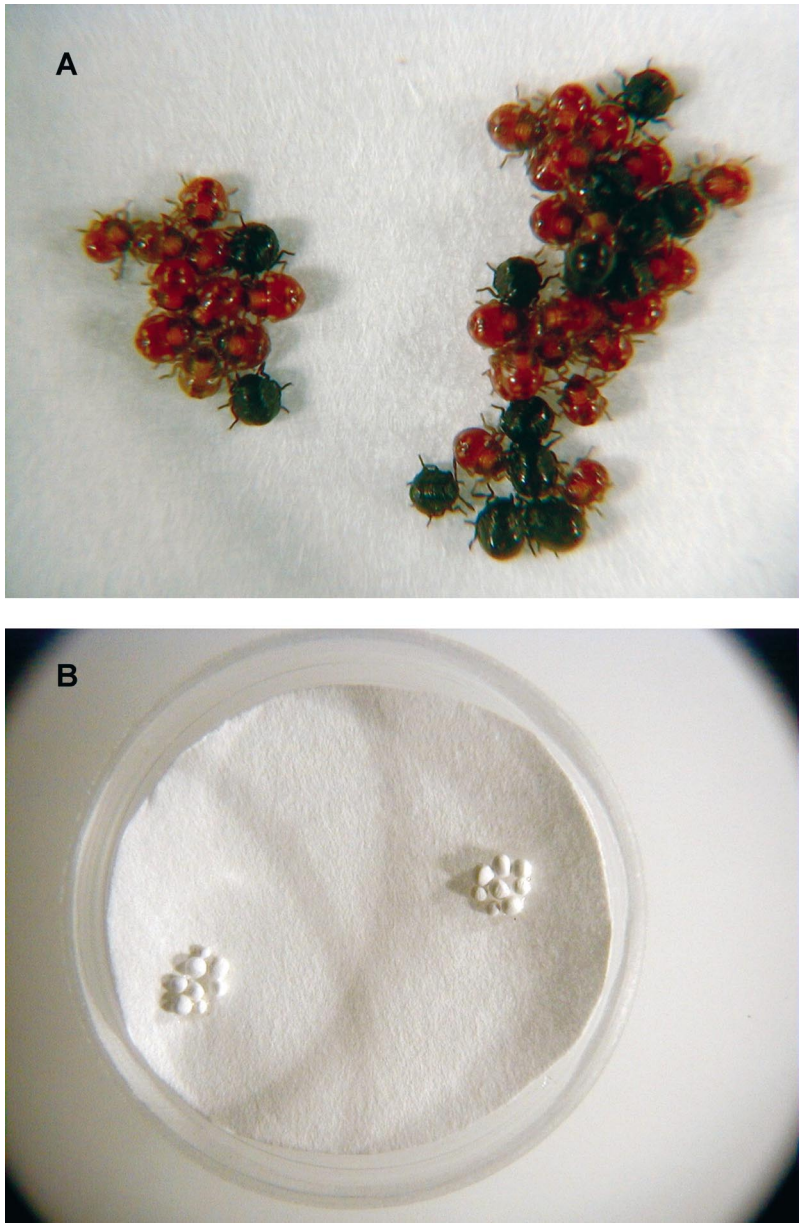

FIG. 2. (A) Example of heterospecific aggregation comprised of first instar nymphs of *N. viridula* (red) and *C. ligata* (black). (B) Bioassay arena (4 cm diam) with groups of polysulfone beads (1 mm diam) mimicking egg masses.

TABLE 3. FORMATION OF HETEROSPECIFIC AGGREGATIONS OF FIRST INSTAR NYMPHS, TESTING ALL POSSIBLE PAIRED COMBINATIONS OF SIX STUDY SPECIES[a]

Species pairs		Number of heterospecific aggregations					Number of nymphs per aggregation (Sp. A–Sp. B)		Mean (± SD) number of nymphs per aggregation at 5 hr	
Sp. A	Sp. B	15 min	30 min	1 hr	2 hr	5 hr	After 15 min	After 5 hr	Sp. A	Sp. B
N.v.	A.h.	3	4	7	4	5	1–1	10–8	4.91 ± 0.66	4.47 ± 0.51
N.v.	C.l.	5	4	4	3	3	1–2	7–6	3.84 ± 0.58	3.15 ± 0.47
N.v.	C.s.	5	5	4	4	3	1–3	7–13	4.90 ± 0.66	4.90 ± 0.84
N.v.	T.p.	4	3	6	5	4	1–1	10–10	4.63 ± 0.62	4.09 ± 0.58
N.v.	E.c.	5	4	2	4	5	2–1	4–9	3.63 ± 0.56	4.05 ± 0.64
A.h.	C.l.	4	4	3	2	2	2–1	14–8	6.20 ± 1.10	5.13 ± 0.67
A.h.	C.s.	6	5	5	3	3	1–1	8–6	4.77 ± 0.72	3.54 ± 0.45
A.h.	T.p.	2	3	4	3	3	1–5	5–8	3.73 ± 0.56	5.06 ± 0.67
A.h.	E.c.	3	5	4	3	3	1–1	3–8	1.94 ± 0.23	4.38 ± 0.69
C.l.	C.s.	5	5	5	4	5	2–1	8–8	4.66 ± 0.40	5.20 ± 0.56
C.l.	T.p.	3	5	4	4	3	1–2	9–8	4.68 ± 0.56	4.52 ± 0.47
C.l.	E.c.	3	4	4	4	2	1–3	5–21	4.64 ± 0.61	5.58 ± 1.40
C.s.	T.p.	3	6	5	7	5	1–1	4–13	4.19 ± 0.38	3.61 ± 0.65
C.s.	E.c.	7	7	5	5	5	2–1	9–8	4.03 ± 0.46	5.27 ± 0.59
T.p.	E.c.	4	3	4	3	2	2–1	5–11	4.00 ± 0.47	4.50 ± 0.77

Note. N.v. = *Nezara viridula*, A.h. = *Acrosternum hilare*, C.l. = *Chlorochroa ligata*, C.s. = *Chlorochroa sayi*, T.p. = *Thyanta pallidovirens*, *E.c.* = *Euschistus conspersus*.

[a] $N = 2$ replicates.

TABLE 4. PERCENTAGES OF FIRST INSTAR NYMPHS IN CONSPECIFIC AND HETEROSPECIFIC AGGREGATIONS 5 HR AFTER COMBINING 40 NYMPHS OF EACH OF TWO SPECIES IN PETRI DISH ARENAS

Species pairs		% nymphs in conspecific aggregations	% nymphs in heterospecific aggregations	*P*
N.v.	A.h.	15.00	60.00	<0.001
N.v.	C.l.	8.75	37.50	0.007
N.v.	C.s.	13.75	47.50	0.006
N.v.	T.p.	26.25	41.25	0.02
N.v.	E.c.	23.75	46.25	0.04
A.h.	C.l.	6.25	37.50	0.002
A.h.	C.s.	31.25	28.75	0.8
A.h.	T.p.	10.25	33.50	0.02
A.h.	E.c.	17.50	55.00	0.009
C.l.	C.s.	6.25	61.25	<0.001
C.l.	T.p.	16.25	51.25	0.005
C.l.	E.c.	5.00	55.00	<0.001
C.s.	T.p.	11.25	52.50	<0.001
C.s.	E.c.	17.50	56.25	0.004
T.p.	E.c.	15.00	62.50	<0.001

Note. All possible species pairs were tested. N.v. = *Nezara viridula*, A.h. = *Acrosternum hilare*, C.l. = *Chlorochroa ligata*, C.s. = *Chlorochroa sayi*, T.p. = *Thyanta pallidovirens*, E.c. = *Euschistus conspersus*.

DISCUSSION

Stink bug secretions serve as chemical defenses in interspecific interactions, but they also have been reported to have a role as intraspecific alarm and aggregation pheromones for nymphs (Lockwood and Story, 1985). First instars of many pentatomid species remain clustered on top of the mass of eggshells from which they have emerged, and if the bugs are dispersed, they rapidly reaggregate (Ishiwatari, 1976). With *N. viridula*, the initial eggshell aggregation is maintained by tactile stimulation, whereas the secondary aggregation is primarily chemically mediated for *N. viridula* and for *Eurydema* spp. (Borges and Aldrich, 1992). Ishiwatari (1976) conducted the first detailed investigations of the possible cues mediating aggregation in first instar cabbage bugs, *Eurydema rugosa*. The main sensory modalities mediating aggregation appeared to be olfaction and touch, because first instar bugs were shown to aggregate preferentially on clusters of small objects (mimicking other bugs) scented with bug extract or with (*E*)-2-hexenal, a prominent component of the *E. rugosa* nymphal extract. Aggregative behavior was eliminated in antennectomized bugs, providing further evidence for the probable role of olfactory and possibly tactile cues (Ishiwatari, 1976).

The results of our studies clarified the role of chemical cues in *N. viridula* aggregation behaviors. In agreement with Borges and Aldrich (1992), we found that

TABLE 5. AGGREGATION OF FIRST INSTAR *N. viridula* ON ARTIFICIAL EGG MASSES TREATED WITH NYMPHAL WHOLE BODY EXTRACT, OR WITH SYNTHETIC COMPONENTS OF THE WHOLE BODY EXTRACT

Treatment	Dose	Mean number (±SD) of nymphs on treatment	Mean number (±SD) of nymphs on control	*P*
Crude whole body extract	Undiluted	0.10 ± 0.69	0.15 ± 0.82	0.66
	50% dilution	0.30 ± 0.15	0.00 ± 0.0	0.055
	10% dilution	0.25 ± 0.11	0.45 ± 0.13	0.26
	1% dilution	0.45 ± 0.15	0.40 ± 0.15	0.85
4-Oxo-(*E*)-2- decenal	50 μg	0.30 ± 0.16	0.80 ± 0.29	0.094
	25 μg	1.20 ± 0.27	0.95 ± 0.42	0.68
	5 μg	4.55 ± 0.60	0.25 ± 0.25	<0.001
	0.5 μg	2.80 ± 0.64	0.40 ± 0.22	0.005
(*E*)-2-Decenal	1000 μg	0.10 ± 0.05	0.85 ± 0.23	<0.001[a]
	100 μg	0.00 ± 0.00	1.40 ± 0.34	0.002[a]
	10 μg	0.30 ± 0.14	4.15 ± 0.43	<0.001[a]
	1 μg	0.15 ± 0.08	2.15 ± 0.67	0.004[a]
Tridecane	1000 μg	1.45 ± 0.36	0.70 ± 0.20	0.13
	100 μg	0.55 ± 0.15	0.75 ± 0.20	0.1
	10 μg	2.11 ± 0.26	2.11 ± 0.43	1.00
	1 μg	1.15 ± 0.38	1.92 ± 0.59	0.15

[a] Compounds significantly repellent.

nymphs did not aggregate in response to whole body extract, and further testing of the individual components of the extract revealed a possible explanation. Whereas 4-oxo-(*E*)-2-decenal induced aggregation at two of the doses tested, in agreement with Pavis et al. (1994), another component of the whole body extract, (*E*)-2-decenal, was repellent at all doses tested, suggesting that (*E*)-2-decenal in whole body extracts may mask or negate the effects of 4-oxo-(*E*)-2-decenal. In contrast to Lockwood and Story (1985), who reported that tridecane was attractive at lower doses and repellent at higher doses, we did not find tridecane to have any effect, either positive or negative, on the behavior of first instar *N. viridula*, even at doses as high at 1000 μg per egg mass mimic.

One of the more surprising discoveries from our experiments was that nymphs of all six study species readily formed interspecific aggregations. In fact, in 14 out of the 15 interspecific combinations tested, the nymphs preferentially formed interspecific aggregations. This phenomenon had been previously noted for two congeneric *Eurydema* species (Ishiwatari, 1976), but we had not anticipated that this effect might cross generic lines so readily. Whereas it can be speculated that *N. viridula* readily forms aggregations with nymphs of *A. hilare*, *T. pallidovirens*, and *E. conspersus*, because all four species produce 4-oxo-(*E*)-2-decenal, it is unclear which cues might be involved in the formation of congeneric aggregations between *N. viridula* and the two *Chlorochroa* spp., which do not produce

4-oxo-(*E*)-2-decenal. Furthermore, the available data suggest that the interspecific aggregation effect is not simply due to mixing individuals of approximately the same shape and size, because *N. viridula* did not form aggregations with *Eurydema rugosa* or *Eurydema pulchra* (Ishiwatari, 1976). The adaptive value of forming heterospecific aggregations is not immediately apparent, but the benefits may be similar to those enjoyed by conspecific aggregations, such as decreased desiccation and decreased predation risk (Kiritani, 1964; Lockwood and Story, 1986). Furthermore, because first instars do not feed, there are no obvious costs in terms of interspecific competition for food resources for first instar bugs that form heterospecific aggregations. Thus, the significant benefits obtained by forming aggregations, coupled with minimal costs, may have selected for plasticity in what constitute acceptable individuals with which to form aggregations.

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CHEMICAL SIGNALING IN A WOLF SPIDER: A TEST OF ETHOSPECIES DISCRIMINATION

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Abstract—Chemical signals from female wolf spiders that elicit exploratory behavior and courtship in males are often assumed to be species-specific, but males of some species court in response to silk cues deposited by closely related heterospecific females. Such is the case with the wolf spiders *Schizocosa ocreata* and *S. rovneri*, ethospecies reproductively isolated on the basis of differences in behavioral mechanisms during courtship. We explored whether male *S. ocreata* and *S. rovneri* reciprocally discriminate species-specific chemical or mechanical cues associated with female silk by using male behavioral response as an assay. Males were exposed to stimulus treatment categories including silk, washed silk, silk extract, and appropriate controls within conspecific or heterospecific female stimulus categories. Male *S. ocreata* and *S. rovneri* did not discriminate between conspecific or heterospecific female stimuli, and courtship intensity was greatest on untreated silk. There were no differences in latency to begin courtship or in rates of courtship behaviors attributed to species origin of silk. However, silk treatment (washed silk, extract) had a significant effect on display and exploratory behaviors (e.g., chemoexplore) in both species. Methanol extraction of female silk successfully removed or inactivated a component necessary to elicit active courtship, but extraction did not significantly reduce exploratory behavior, suggesting that a separate compound may be responsible for releasing this behavior. Together, these experiments support the characterization of *S. ocreata* and *S. rovneri* as ethospecies reproductively isolated only by female discrimination of species-specific male courtship, and indicate that chemical, but not mechanical cues associated with silk are critical for eliciting male courtship in both species.

Key Words—Behavioral assay, chemical communication, *Schizocosa*, Lycosidae, wolf spider, species discrimination.

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INTRODUCTION

Spiders, like most arthropods, use chemical signals to mediate many types of interactions. These interactions may include prey detection, predator avoidance, social recognition, and courtship (reviews in Tietjen and Rovner, 1982; Uetz, 2000; Barth, 2002; Uetz and Roberts, 2002). Male spiders may produce compounds that alter the behavior of conspecifics, either in the context of male/male competition by suppression of activity in other males (Tietjen, 1979; Ayyagari and Tietjen, 1987), or in mating behavior by either releasing female receptivity displays (Ross and Smith, 1979) or inducing a cataleptic state in females prior to copulation (Singer et al., 2000). However, most studies of chemical signaling in spiders have focused on compounds associated with the silk and/or cuticle of females and the role these putative pheromones play in mate attraction and release of exploratory and courtship behaviors (Tietjen and Rovner, 1982; Pollard et al., 1987; Riechert and Singer, 1995; Trabalon et al., 1997; Prouvost et al., 1999; Papke et al., 2001).

In wolf spiders (Araneae: Lycosidae), there is evidence that females can attract males with airborne chemical signals (Tietjen, 1979; Searcy et al., 1999), but most chemical signaling appears to be via contact chemical compounds associated with silk. Conspecific female silk and associated pheromones are sufficient to elicit normal courtship behavior in males of many species, even in the absence of all visual and vibratory cues (den Hollander et al., 1973; Tietjen and Rovner, 1982; Stratton and Uetz, 1983; Costa and Capocasale, 1984). While the chemical signals from female wolf spiders that elicit exploratory behavior and release courtship in males are thought to be species-specific (Hegdekar and Dondale, 1969; Tietjen, 1977; Costa and Capocasale, 1984), males of closely-related species will sometimes court the silk of heterospecifics (Kaston, 1936; Hegdekar and Dondale, 1969; den Hollander, 1971; Miller et al., 1998).

Such is the case with males of the two wolf spider species used in this study, *Schizocosa ocreata* (Hentz) and *S. rovneri* (Uetz and Dondale). These species can be considered "ethological species" (Dobzhansky, 1970) and have been described as "ethospecies" (Uetz and Denterlein, 1979), that is, species that are isolated reproductively only on the basis of differences in behavioral mechanisms occurring during courtship (den Hollander and Dijkstra, 1974), with no other prezygotic isolating mechanisms (Uetz and Denterlein, 1979; Stratton and Uetz, 1981, 1983, 1986). *Schizocosa ocreata* and *S. rovneri* prefer different microhabitats but overlap in geographical range and habitat use (Uetz and Denterlein, 1979; Stratton and Uetz, 1981; McClintock and Uetz, 1996), and are essentially seasonally synchronous (Uetz and Denterlein, 1979; Roberts, J. A., unpublished data). Male *S. ocreata* do not show evidence of territoriality, and may travel great distances each day during some portions of the breeding season (linear distance of up to 15 m/hr—Roberts, J. A. and Uetz, G. W., unpublished data), increasing chances of encounter

with both conspecifics and heterospecifics. In fact, males allocate much of their time to searching for females in the complex habitat (Cady, 1984).

There is no evidence of mechanical or gametic incompatibility, as hybrids of these two species have been successfully produced in "forced" copulation studies in laboratory experiments (Stratton and Uetz, 1986; Orr, M. and Uetz, G. W., unpublished data). The genes that code for courtship behavior do not assort independently so that otherwise viable hybrids suffer behavioral sterility, and no known natural hybrids have yet been identified (Stratton and Uetz, 1986). Females of these species are receptive only to species-specific visual and vibratory courtship cues (Uetz and Denterlein, 1979; Stratton and Uetz, 1981, 1983, 1986; but see Uetz and Roberts, 2002), a finding that is not surprising as females mating with heterospecifics would suffer fitness consequences related to behaviorally sterile offspring.

Males of both species have been demonstrated to be equally likely to court both conspecific and heterospecific silk (Uetz and Denterlein, 1979; Stratton and Uetz, 1983), and *S. ocreata* males court with similar intensity on both silks (Roberts and Uetz, unpublished data), but there has not been a rigorous, reciprocal test of male discrimination of female cues. Male courtship is costly in these species, with recent studies indicating a high energetic expense (Cady, A. B., Uetz, G. W., and Delaney, K. J., unpublished data), and, for *S. ocreata*, that courtship characteristics important in mate choice also increase predation (Roberts, J. A., Taylor, P. W., and Uetz, G. W., unpublished data). Intraguild predation has a large impact on survival in wolf spiders (Polis and McCormick, 1986; Wise and Chen, 1999), and males making discrimination mistakes may become prey if they court heterospecifics.

We predict that if there are detectable differences in female cues associated with silk, there should be differences in the intensity of courtship (i.e., rate of behaviors) for males of both species when exposed to conspecific versus heterospecific cues. In addition, male courtship response is thought to be elicited by chemical compounds associated with silk (contact pheromones), but this has not been confirmed using silk extractions. While unlikely, it is possible that male courtship response in these species could be due to mechanical properties of silk. In this study, we explored whether males are able to discriminate species-specific chemical cues associated with female silk, testing the null hypothesis of no behavioral differences (nondiscrimination). Any significant differences in courtship intensity would suggest species discrimination and could indicate species recognition via chemical or mechanical cues associated with silk.

METHODS AND MATERIALS

General Methods. The wolf spider ethospecies used in this study are common in deciduous forest habitats of eastern and mid-western North America.

All spiders used in this study were collected as juveniles or subadults in the field and then raised to adulthood under identical laboratory conditions. Juveniles and adult females of these species are morphologically indistinguishable. Adult males of each species can be identified by the presence/absence of large tufts of bristles found only on the forelegs of *S. ocreata*. As the experiments described here required prior knowledge of species identity, all individuals were collected from unique species-pure localities surrounded by the zone of sympatry. *Schizocosa ocreata* were collected from the Cincinnati Nature Center, Rowe Woods, Clermont County, Ohio, and the University of Cincinnati Benedict Nature Preserve, Hamilton County, Ohio. *Schizocosa rovneri* were collected from the Ohio River flood plain at Sandy Run, Boone County, Kentucky. Individuals were visually isolated from other spiders in opaque plastic containers (deli-dish, 10-cm diam), fed (as appropriate for their size/age) assorted collembolans, fruit flies (*Drosophila*), or two–three 10-day-old cricket nymphs (*Acheta domesticus*) twice weekly, and supplied with water *ad libitum* from a cotton wick suspended in a water reservoir below the container. All spiders were maintained at room temperature (22–25°C) with stable humidity and a 13:11-hr light:dark photoperiod. Males and females were unmated and ranged from 1 to 4-wk postfinal molt at the time of the study.

Collection of Silk and Extraction of Chemical Cues. Female *S. ocreata* deposit dragline silk as they walk, so in treatments where female silk was necessary for study, it was collected by placing single females on filter paper (Fisherbrand® 90-mm diam) in a glass petri-dish (90-mm diam) allowing each to locomote for 24 hr and deposit dragline silk. Females were not used on the same day they had been fed to reduce contamination from feces (Ayyagari and Tietjen, 1987). Fecal material is quickly absorbed by filter paper allowing any fecal stains to easily be excised prior to chemical extraction or use in experiments. Filter paper was handled with stainless steel forceps, and stains were excised by using a stainless steel dissecting blade. Any filter paper disk with three or more fecal stains was not used in this study. To reduce the likelihood of contamination, silk collection equipment was cleaned in Alconox® detergent and rinsed in double deionized water and 70% ethanol between filter paper manipulations.

Preliminary behavioral assays were conducted with *S. ocreata* to identify an effective solvent for extraction of chemical cues associated with female silk. We exposed males (5 min) to three stimulus treatments including untreated silk ($N = 10$) and silk treated in 3-min extractions with pentane ($N = 12$, Fisher Scientific, HPLC grade) or methanol ($N = 10$, Pharmco Products, Inc., HPLC grade). Each of these solvents has been used previously to successfully extract behaviorally active chemical components associated with spider silk (Ayyagari and Tietjen, 1987; Trabalon et al., 1997). We used total duration of active courtship (jerky tap) exhibited by male *S. ocreata* on washed silk as a measure of extraction effectiveness. Methanol was the best solvent, and this result was consistent with a previous

study in which male *S. ocreata* pheromone compounds were extracted (Ayyagari and Tietjen, 1987).

For the collection of silk, 120 females (60 of each of the two ethospecies) were used. The female stimulus treatments for the *S. ocreata* and *S. rovneri* discrimination experiments were prepared as follows; (1) *Untreated Silk*– filter paper disks containing silk (and associated chemical cues) of conspecific or heterospecific females were used directly to elicit male behavior, (2) *Washed Silk*– following silk collection, filter paper disks with bound silk were soaked in 50 ml of methanol for 30 min, removed from the solvent bath, and allowed to air-dry for 30 min, and (3) *Silk Extract*– filter papers were soaked in 50 ml of methanol for 30 min, discarded, and the remaining solvent allowed to completely evaporate on a clean piece of filter paper for 30 min. Previous experiments involving the extraction of silk for behavioral assays have used extraction periods ranging from 15 min (Jackson, 1987) to 180 min (Suter and Renkes, 1982). Ayyagari and Tietjen (1987) found that a 15-min extraction in only 10 ml of solvent was sufficient to remove behaviorally active components from the silk of male *S. ocreata*, but we chose 30 min in 50 ml of solvent (enough to completely submerge the filter paper and silk) as a more conservative estimate to ensure more complete extraction (Suter, R. B., personal communication). The *Untreated Control* (4) treatment (filter paper alone) was prepared by duplicating the silk collection treatments but without female silk, including placing each filter paper in a glass petri-dish for 24 hr. All prepared filter paper disks were used in behavioral trials within 4 hr of completion of the preparation.

Behavioral Assays. We used male behavioral response to chemical and/or mechanical stimuli as an assay of male discrimination of female species identity. Behavioral trials were conducted during the period of peak activity for males (1000–1600 hr, Roberts, J. A. and Uetz, G. W., unpublished data), in clear plastic containers ($13 \times 7 \times 7$ cm) that provided a visual arena suitable for videotaping. Filter paper disks were handled only with fine, stainless steel forceps during all stages of pre- and posttreatment manipulation. Each disk was cut in half with stainless steel dissecting scissors, and both halves were placed into the arena no more than 5 min prior to the start of a trial. Males were placed gently onto the filter paper stimulus from above and videotaped (RCA Autoshot® VHS video camera, Model CC4352) for later behavioral analysis with a software package for the detailed analysis of behavioral data (The Observer® , version 4.1). The plastic arenas, dissecting scissors, and forceps were cleaned prior to each trial with a Kimwipe® and 70% ethanol to remove any trace of silk and pheromone deposited in previous trials and were allowed to air-dry.

A total of 160 males (80 of each species) were chosen randomly from the laboratory population and assigned to one of four stimulus treatment groups (described above) within the species categories *S. ocreata* or *S. rovneri* (a two-factor ANOVA with treatment and species as main effects and individuals as replicates).

TABLE 1. ETHOGRAM OF MALE BEHAVIORAL ELEMENTS FOR THE WOLF SPIDERS *Schizocosa ocreata* AND *Schizocosa rovneri*[a]

Schizocosa ocreata	
Jerky tap	Active courtship characterized by a combination of jerky forward walking motion and tapping of the forelegs against the substrate, and often ending with the chelicerae striking the substrate.
Tap	One or both forelegs are raised off the substrate and then lowered, lightly striking the substrate.
Schizocosa rovneri	
Bounce	While stationary, all legs are contracted simultaneously causing the ventral surface of the body to strike the substrate.
Shared behaviors	
Chemoexplore	Active exploratory behavior where the anteriolateral surfaces of the pedipalps are brushed on the surface of the substrate in rapid succession.
Leg raise	One or more legs are raised off the substrate above parallel with the body and then lowered without striking the substrate. This is a combination of "arch" (Stratton and Uetz, 1986) and all other leg waving.
Grooming	The legs or pedipalps are drawn between the chelicerae and/or lateral pairs of legs are brushed together rapidly.
Locomotion	Walking with no other elements expressed.
Stationary	Motionless with no other elements expressed.

[a]Behavioral elements have been adapted from Stratton and Uetz (1986) and Delaney (1997).

Shared and species-specific male behavioral elements were scored for each trial (Table 1). The behaviors critical in discrimination and courtship in these species are chemoexplore (both species), jerky tap (male *S. ocreata*), and bounce (male *S. rovneri*) (Stratton and Uetz, 1986; Stratton, 1997; see ethogram, Table 1), and results for these behaviors are presented for each species.

Two parameters were determined for each key behavior; (1) *Total Number* – a count of the number of bouts (or events) of each behavior during the trial period, and (2) *Latency* – the time from introduction of the male into the arena to the initiation of a behavior (a score of 300 sec was used if a behavior was not seen during the 5-min trial period). The active courtship element of *S. rovneri* ("bounce") occurs as an event (an instantaneous behavior with each occurrence having a duration less than 1 sec; measured in terms of rate) and not a behavioral state (behavioral elements with a measurable duration), so parameters including a term for duration of behavior could not be calculated for *S. rovneri* courtship and were not calculated for either species. Each parameter scored was square root transformed for analysis, and for each species a separate, Bonferroni-corrected critical value ($\alpha = 0.025$) was used in significance tests of the ANOVA results to account for multiple comparisons (Shaffer, 1995). *Post hoc* analyses of the preliminary assay results and pooled data from the behavioral assays, where appropriate, were made

with Tukey–Kramer HSD tests (Zar, 1999). All statistical analyses were performed with JMP version 4.02 (SAS Institute).

RESULTS

Preliminary Assay. Total duration of male courtship behavior on washed silk varied significantly with extraction treatment (ANOVA, $F_{2,29} = 9.4$, $P < 0.001$). Male *S. ocreata* behavior on female silk washed in pentane was not significantly different from untreated silk, but behavior was significantly reduced on silk washed in methanol.

Schizocosa ocreata, Chemoexploratory Behavior. The total number of bouts and latency of chemoexploratory behavior both varied significantly with stimulus treatment, but there was no effect of species (conspecific or heterospecific source of silk) on male behavior, and no interaction between factors (Figure 1). After pooling the species data for each treatment group, we found that males exhibited the most bouts of chemoexploratory behavior on untreated and washed silk, with frequency of bouts significantly reduced on silk extract, and lowest in the control treatments (Figure 1A). Latency to chemoexplore was shortest on untreated silk followed by washed silk, then silk extract, and longest in the control treatment (Figure 1B).

Schizocosa ocreata, Courtship Behavior. As in the data for chemoexploratory behavior, the total number and latency of courtship bouts in *S. ocreata* varied significantly with stimulus treatment, and there was again no significant effect of the source of silk (species) and no significant interaction (Figure 2). The number of bouts of jerky tap was highest on untreated silk, significantly reduced in response to washed silk, and not different from untreated control for the extract treatment (Figure 2A). Males began actively courting on untreated silk faster than on any of the other stimulus treatments (Figure 2B).

Schizocosa rovneri, Chemoexploratory Behavior. For *S. rovneri*, both the total number and latency of bouts of chemoexploratory behavior varied with stimulus treatment, and while there was no effect of species for either parameter, the interaction was significant for total number of bouts of chemoexplore behavior (Figure 3). This interaction, presumably due to response in the silk extract treatment where the species difference is not consistent with the pattern of differences in other treatments, precluded a *post hoc* analysis of number of bouts of chemoexplore behavior by treatment stimulus (Figure 3A). Latency to chemoexplore was shortest on untreated silk, intermediate on washed silk and silk extract, and longest for the control treatment (Figure 3B).

Schizocosa rovneri, Courtship Behavior. The total number of bounce bouts was significant by treatment, as was the latency of bounce behavior, with no effect of species or interaction term for either measure (Figure 4). Males exhibited more

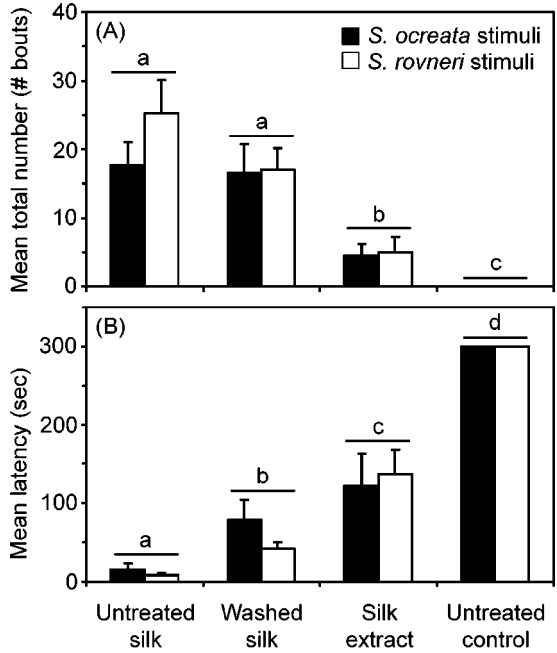

FIG. 1. *Schizocosa ocreata* chemoexploratory behavior. For male spiders exposed to each treatment within the conspecific (*S. ocreata*) or heterospecific (*S. rovneri*) species categories; (A) mean total number of behavioral bouts (+SE) (ANOVA, treatment: $F_{3,72} = 40.2$, $P < 0.001^a$; species: $F_{1,72} = 0.8$, $P = 0.365$; interaction: $F_{3,72} = 0.4$, $P = 0.748$), and (B) mean latency (*s*) of behavioral bouts (+SE) (ANOVA, treatment: $F_{3,72} = 64.5$, $P < 0.001^a$; species: $F_{1,72} = 0.0$, $P = 0.829$; interaction: $F_{3,72} = 0.7$, $P = 0.560$) (^{a}Indicates significance using Bonferroni-corrected critical value $\alpha = 0.025$ to account for multiple comparisons). Shared letters above the bars indicate no significant difference between treatment categories by Tukey–Kramer HSD post hoc analysis (with species category data pooled).

bounce bouts on untreated silk than on any other treatment (Figure 4A), and latency to begin bounce behavior was shortest on untreated silk and not different from untreated control for the other treatments (Figure 4B).

DISCUSSION

According to Dobzhansky (1970), "ethological species" are those in which there is a breakdown in some component of mutual attraction between the sexes in heterospecific pairings that prevents reproduction. In spiders, at least, species that meet this definition have been called "ethospecies" (den Hollander and Dijkstra, 1974; Stratton and Uetz, 1981, 1983, 1986). Stratton and Uetz (1981) presented

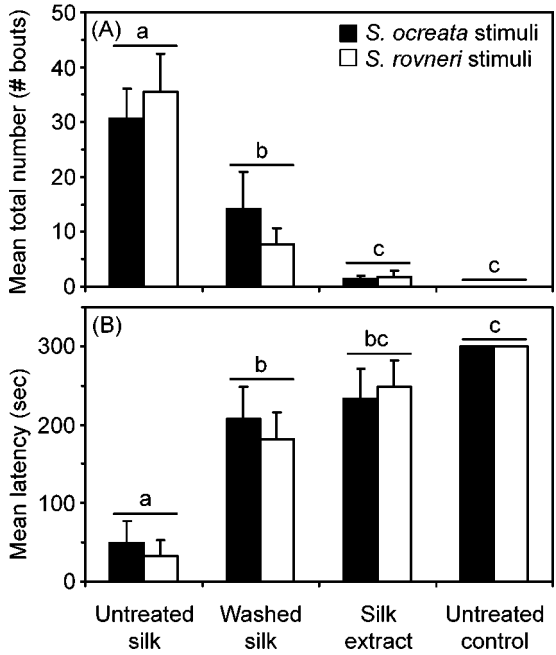

FIG. 2. *Schizocosa ocreata* jerky tap behavior. For male spiders exposed to each treatment within the conspecific (*S. ocreata*) or heterospecific (*S. rovneri*) species categories; (A) mean total number of behavioral bouts (+SE) (ANOVA, treatment: $F_{3,72} = 33.8$, $P < 0.001^a$; species: $F_{1,72} = 0.0$, $P = 0.999$; interaction: $F_{3,72} = 0.1$, $P = 0.933$), and (B) mean latency (*s*) of behavioral bouts (+SE) (ANOVA, treatment: $F_{3,72} = 31.6$, $P < 0.001^a$; species: $F_{1,72} = 0.0$, $P = 0.887$; interaction: $F_{3,72} = 0.2$, $P = 0.923$) (^{a}Indicates significance using Bonferroni-corrected critical value $\alpha = 0.025$ to account for multiple comparisons). Significant differences indicated as in Figure 1.

evidence of reproductive isolation between *S. ocreata* and *S. rovneri* based on species-specific male courtship behaviors without a rigorous test of reciprocal chemical cue discrimination. In this study, males of both *S. rovneri* and *S. ocreata* responded equivalently with respect to courtship and exploratory behaviors on untreated conspecific and heterospecific female silk cues. It appears that either there are no differences between female signals, or that there are no species-specific differences recognizable by males of either species. Therefore, female discrimination of species-specific male courtship appears to be the only component of attraction preventing reproduction in these ethospecies, supporting previous studies (Stratton and Uetz, 1981, 1983, 1986). The results of experiments presented here also suggest that chemical, and not mechanical cues associated with silk are critical for eliciting male courtship in both *S. ocreata* and *S. rovneri* (Uetz

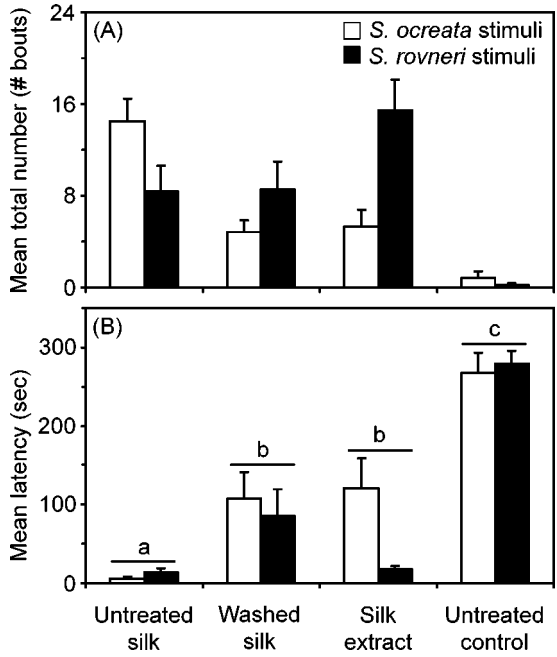

FIG. 3. *Schizocosa rovneri* chemoexploratory behavior. For male spiders exposed to each treatment within the conspecific (*S. rovneri*) or heterospecific (*S. ocreata*) species categories; (A) mean total number of behavioral bouts (+SE) (ANOVA, treatment: $F_{3,72} = 25.9$, $P < 0.001^a$; species: $F_{1,72} = 1.6$, $P = 0.208$; interaction: $F_{3,72} = 7.0$, $P < 0.001^a$), and (B) mean latency (s) of behavioral bouts (+SE) (ANOVA, treatment: $F_{3,72} = 45.0$, $P < 0.001^a$; species: $F_{1,72} = 1.7$, $P = 0.195$; interaction: $F_{3,72} = 3.0$, $P = 0.036$) (^{a}Indicates significance using Bonferroni-corrected critical value $\alpha = 0.025$ to account for multiple comparisons). The significant interaction effect in (A) prohibited a *post hoc* analysis by treatment category, however significant differences for (B) indicated as in Figure 1.

and Denterlein, 1979; Stratton and Uetz, 1983). Even so, while we were able to successfully remove a compound on female silk that elicits male courtship, extracts were not sufficient to stimulate males to court at levels equivalent to untreated silk. There may be more than one potential explanation for this finding.

One possible explanation relates to stimulus strength. Ayyagari and Tietjen (1987) extracted behaviorally active components from male silk in *S. ocreata*, but in that study, the silk of multiple individuals was extracted simultaneously to arrive at a product that would elicit male response. This method has the undesirable side effect of potentially creating a "super-stimulus" (a chemical stimulus far stronger than any that would be encountered in the natural habitat) that could result in atypical behavioral response in stimulated individuals. In an attempt to arrive at

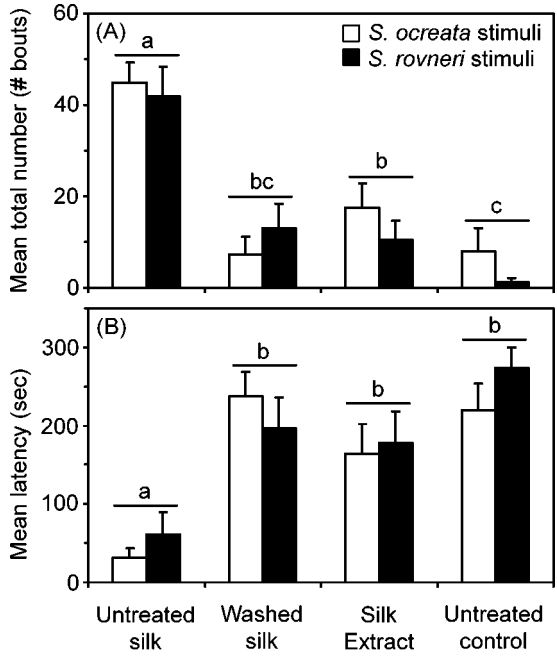

FIG. 4. *Schizocosa rovneri* bounce behavior. For male spiders exposed to each treatment within the conspecific (*S. rovneri*) or heterospecific (*S. ocreata*) species categories; (A) mean total number of behavioral bouts (+SE) (ANOVA, treatment: $F_{3,72} = 23.8$, $P < 0.001^a$; species: $F_{1,72} = 0.9$, $P = 0.346$; interaction: $F_{3,72} = 1.0$, $P = 0.391$), and (B) mean latency (s) of behavioral bouts (+SE) (ANOVA, treatment: $F_{3,72} = 17.3$, $P < 0.001^a$; species: $F_{1,72} = 0.3$, $P = 0.599$; interaction: $F_{3,72} = 0.8$, $P = 0.512$) (^{a}Indicates significance using Bonferroni-corrected critical value $\alpha = 0.025$ to account for multiple comparisons). Significant differences indicated as in Figure 1.

more natural dose levels, we used extracts of the silk of single individuals. It seems likely that the low courtship response in our study may have been due to diffusion of a finite amount of chemical stimulus over a greater area, reducing point-specific concentration to levels below the threshold necessary for release of courtship.

Another possibility is that there may be multiple compounds affected differently by solvent type. Ayyagari and Tietjen (1987) used methanol to extract a behaviorally active component from male *S. ocreata* silk, and in part for this reason methanol was selected as a potential solvent for female chemical compounds in the work presented here. In previous attempts to study potential pheromones associated with the silk of female wolf spiders, several organic and inorganic solvents were found to produce a pattern of inactivation similar to that found using methanol in the current study (Kaston, 1936; Hegdekar and Dondale, 1969). This evidence

is suggestive that male courtship response is elicited by multiple compounds that are differentially extracted in various solvents but are necessary in combination to elicit courtship.

In contrast to the results for male courtship, males exhibited a similar number of bouts of chemoexploratory behavior on untreated silk and silk washed in methanol (Figures 1 and 3). This suggests that the chemical compound(s) that elicits courtship behavior plays only a limited role for release of chemoexploratory behavior. Either there is a separate compound associated with silk that releases chemoexploratory behavior but is not extracted by methanol, or the term "chemo" exploratory behavior may be a misnomer for these species. Wolf spiders are known to have chemosensory sensilla concentrated on the anterior dorsal and lateral surfaces of the pedipalps (Kronestedt, 1979; Tietjen and Rovner, 1982; Barth, 2002), which are actively rubbed on the substrate during exploratory behavior presumably to collect chemical information (Tietjen, 1977; Stratton and Uetz, 1986). In another study of *S. ocreata* (Roberts, J. A. and Uetz, G. W., unpublished data), total number of bouts of chemoexploratory behavior was similar on the silk of female *S. crassipes*, a member of the *ocreata* clade along with *S. ocreata* and *S. rovneri* (Miller et al., 1998), but decreased in species outside the clade despite the presence of silk. Together this evidence suggests that a chemical associated with silk elicits chemoexploration, and that this compound may be independent of, or redundant to, the chemical that releases courtship behavior. Of note is the unexpected result for *S. rovneri* males of greater number of bouts of chemoexploratory behavior on extract of heterospecific silk, compared to conspecific extract (Figure 3A). This suggests that there may be slight differences in the composition or ratios of chemical compounds on the silk of these species, but those differences are not behaviorally relevant on untreated silk. Further chemical analysis will be necessary to fully explain this difference.

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FLORAL CO_2 REVEALS FLOWER PROFITABILITY TO MOTHS

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Abstract—The hawkmoth *Manduca sexta* (Lepidoptera: Sphingidae), an experimentally favorable Lepidopteran that is highly sensitive to carbon dioxide (CO_2), feeds on the nectar of a range of flowering plants, such as *Datura wrightii* (Solanaceae). Newly opened *Datura* flowers give off dramatically elevated levels of CO_2 and offer ample nectar. Thus, floral CO_2 emission could indicate food-source profitability. This study documents that foraging *Manduca* moths prefer surrogate flowers that emit high levels of CO_2, characteristic of newly opened *Datura* flowers. We show for the first time that CO_2 may play an important role in the foraging behavior of nectar-feeding insects.

Key Words—*Manduca sexta*, *Datura wrightii*, CO_2, labial-palp pit organ, insect-plant interactions, foraging.

INTRODUCTION

While it is well established that haematophagous insects use CO_2 to locate their hosts (Stange, 1996), its relevance for herbivorous insects remains mostly elusive. Intriguingly, however, adult herbivorous Lepidoptera have especially well developed CO_2 sensory organs (Stange, 1996; Stange and Stowe, 1999). Thus, the experimentally favorable sphinx moth *Manduca sexta* (hereinafter *Manduca*) and other investigated moths that feed as adults have large labial-palp pit organs (LPOs) that sensitively detect CO_2 (Kent et al., 1986; Stange, 1996; Guerenstein et al., 2002), while at least some species that do not feed as adults have reduced LPOs (Kent et al., 1986). Furthermore, newly opened and highly profitable flowers of *Datura wrightii* (Solanaceae), a preferred hostplant of *Manduca*, emit significantly

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elevated levels of CO_2 (Thom et al., 2003; Guerenstein et al., 2004). Exploited, unprofitable flowers are likely to have less CO_2 as feeding moths may fan CO_2 out of the flower. We hypothesized that nectar-feeding moths use floral CO_2 cues to identify profitable flowers and to increase their foraging efficiency by preferably targeting these profitable flowers. To test this idea, we recorded in a dual-choice experiment whether foraging *Manduca* moths prefer to extend their proboscis into a scented, non-rewarding (nectar-free) surrogate flower that emits (a) an approximately ambient level of CO_2 or (b) the higher level of a newly opened *Datura* flower.

METHODS AND MATERIALS

Experiments were performed in April and May, 2003. *Manduca sexta* larvae were reared under standard conditions (Tolbert et al., 1983), and transferred into a growth chamber (Percival Scientific, Inc., Perry, IA) at least 3 d before eclosion. The chamber provided a reversed light cycle with darkness from 0800 to 2000 hr, and temperatures of 25°C and 27°C, respectively. Choice experiments were performed with unmated male moths to avoid potential interference of oviposition behavior.

Prior to the choice experiment, we established with electrophysiological methods that the LPOs of male *Manduca* sensitively detect CO_2, as was reported for females (Guerenstein et al., 2002), and that the antennae of neither sex respond to CO_2.

To test whether *Manduca* moths prefer to feed from high-CO_2 flowers, foraging moths in a laboratory flight cage ($1.8 \times 1.8 \times 1.8$ m) were given a choice between two surrogate flowers with different levels of CO_2. Surrogate flowers were made of white cotton paper (Southworth Co., Agawam, MA) and scented with 20 μl of the essential oil of *Asclepia tuberosa* (Asclepiadaceae) flowers diluted to 10^{-3} in odorless mineral oil. Neither sugar, water, nor other rewards were provided to feeding moths. One flower emitted a stream of air carrying 765 ppm CO_2, thus resembling the CO_2 level in the entrance of a newly opened *Datura* flower (Guerenstein et al., 2004), and the other flower, an airstream with 438 ppm CO_2. Both airstreams were delivered through Teflon® tubing and air diffusers at a flow of ca. 35 ml/min in the flower. The average CO_2 background concentration inside the cage during experiments was ca. 480 ppm. Each paper flower was attached to a different *Datura* plant (lacking natural flowers), and plants were spaced 1 m apart in the flight cage. We controlled for effects of individual plants and location by regularly exchanging the gas-delivering tubing among plants.

To determine innate foraging preference, we recorded the target of the first proboscis extension of 19 'naive' moths (1–8 d old) that had not previously extended their proboscis into a surrogate flower and, thus, had no prior foraging experience. To determine whether 'experienced' moths choose differently from

naïve moths, we tested 7 moths (3–6 d old) 24 or 48 hr after the first experiment during which they had extended the proboscis into at least one surrogate flower. To record the target of the first proboscis extension, one moth at a time was released into the flight cage and observed until it stopped flying. The moth was then removed from the cage, and after an interval of at least 5 min., another moth was released. Paper flowers were renewed after five moths were tested, or whenever flowers seemed affected.

RESULTS AND DISCUSSION

Most foraging, naïve moths (95%, $N = 19$) directed their first proboscis extension into the surrogate flower emitting the higher level of CO_2 (Figure 1). Thus, this study shows that the herbivorous moth *Manduca* uses floral CO_2 cues to make foraging decisions. A preference for flowers with high levels of CO_2 should increase foraging success of moths, as natural flowers with high CO_2 are likely to have high amounts of nectar and thus be profitable (Thom et al., 2003; Guerenstein et al., 2004). Because moths that feed as adults have well developed CO_2-sensing organs, while non-feeding species do not (Kent et al., 1986), our findings likely apply to other adult-feeding lepidopteran species as well. Thus, CO_2 appears to play an important role in the foraging behavior of moths.

Experienced *Manduca* moths did not maintain their initial preference for high-CO_2 flowers. Moths that were tested a second time 24 or 48 hr after the first experiment ($N = 7$) no longer preferred the flower with the higher level of CO_2

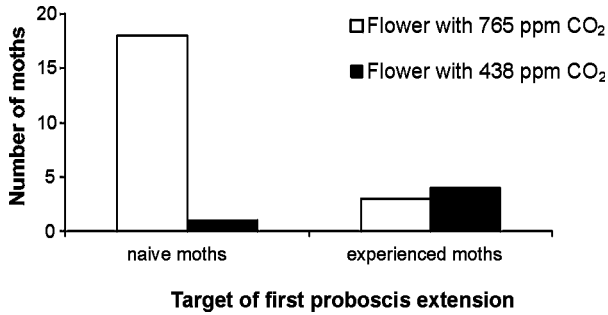

FIG. 1. Targets of the first proboscis extensions (feeding attempts) of naïve and experienced *Manduca* moths: number of moths targeting the surrogate flower with a high level of CO_2 as typical for newly opened *Datura* flowers (765 ppm, white bars), and an approximately ambient level of CO_2 (438 ppm, black bars). The position of flowers in the room did not affect the outcome of experiments. Only the first proboscis extension of a moth during each experiment was considered for analysis of foraging preference, as the first experience might affect successive foraging decisions.

(Figure 1). This suggests that their first, negative foraging experience affected their initial preference for CO_2 (Yates corrected Chi^2-test of independence, $P = 0.016$). A flexible response to floral CO_2 may enable *Manduca* to exploit not only established and possibly co-evolved hostplants such as *Datura*, but also plants in which CO_2 does not indicate floral profitability.

By documenting a foraging function of floral CO_2 cues for herbivorous insects, this study reveals a novel, basic aspect of insect foraging strategies and plant-insect interactions. This is especially interesting as many herbivorous insects are agriculturally important as pests and/or as pollinators. Further, our findings may also help to assess potential effects of the predicted global climate change (IPCC, 2001), as the continuous increase of atmospheric levels of CO_2 has raised much concern about the consequences for insects (Stange and Wong, 1993; Stange, 1997; Percy et al., 2002).

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DIRECT DEFENSE OR ECOLOGICAL COSTS: RESPONSES OF HERBIVOROUS BEETLES TO VOLATILES RELEASED BY WILD LIMA BEAN (*Phaseolus lunatus*)

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Abstract—In response to feeding damage, Lima bean releases herbivore-induced plant volatiles (HIPV), which are generally assumed to attract carnivorous arthropods as an indirect defense. While many studies have focused on such tritrophic interactions, few have investigated effects of HIPV on herbivores. I used natural herbivores of wild Lima bean and studied their responses to jasmonic acid-induced plants in an olfactometer and in feeding trials. Both *Cerotoma ruficornis* and *Gynandrobrotica guerreroensis* (Chrysomelidae) significantly preferred control plants to induced ones in the olfactometer, and they avoided feeding on induced plants. In contrast, Curculionidae significantly preferred HIPV of the induced plant to those of the control in one plant pair and did not choose in the case of a second pair. In feeding trials, no choice occurred in the first plant pair, while control leaves were preferred in the second. Release of HIPV deterred Chrysomelid herbivores and, thus, acted as a direct defense. This may be an important addition to indirect defensive effects. Whether or not HIPV released by induced plants attracted herbivorous Curculionidae, thus incurring ecological costs, varied among plants. Such differences could be related to various HIPV blends released by individual plants.

Key Words—Indirect defense, induced plant volatiles, plant-herbivore interactions, tritrophic interactions, Mexico.

INTRODUCTION

In response to feeding damage, many plants release herbivore-induced plant volatiles (HIPV), which generally are assumed to attract predators or parasitoids

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and, thus, have the potential to act as an indirect defense. However, HIPV are likely to affect the herbivores' behavior too. HIPV released by herbivore-infested plants may deter further herbivores and thereby act as a direct defense (Dicke and van Loon, 2000; De Moraes et al., 2001; Kessler and Baldwin, 2001), but HIPV may also attract herbivores and thereby incur ecological costs (Bolter et al., 1997; Dicke and van Loon, 2000; Kalberer et al., 2001; Horiuchi et al., 2003). Thus, the ecological role of HIPV may differ among plant and herbivore species.

In this study, herbivore behavior toward induced wild Lima bean (*Phaseolus lunatus* L., Fabaceae) plants was investigated in olfactometer and feeding experiments. Lima bean releases several HIPV, a response that is dependent on the octadecanoid pathway. A transient increase in endogenous jasmonic acid (JA) is involved in the synthesis of HIPV (Koch et al., 1999), and volatile blends similar to those of herbivore-damaged plants are released in response to exogenous JA application (Boland et al., 1995; Dicke et al., 1999). Most studies on effects of HIPV released by Lima bean have focused on responses of carnivorous arthropods. Here, I investigated responses of naturally occurring beetles to volatiles released by JA-treated plants in order to better understand how Lima bean interacts with its herbivores.

METHODS AND MATERIAL

Animals and Plant Material. Adult beetles were collected in the field *c.* 10 km w of Puerto Escondido (coastal area of Oaxaca, Mexico) in the first two weeks of December 2003. Beetles appearing on Lima bean were collected to represent natural ratios of sexes and ages. The beetles were kept in 250 ml plastic cups for 1–3 d with water only (water supplied on cotton, but no food) and then subjected to feeding trials or olfactometer experiments. The beetles used were *Cerotoma ruficornis* (Olivier) and *Gynandrobrotica guerreroensis* Jacoby (Chrysomelidae: Galerucinae: Luperini: Subtribe Diabroticina), and a species of Curculionidae. During November and December 2002 and 2003, these species were the most abundant herbivores on Lima bean at the study site. They were present all day long, exhibiting two peaks of feeding and moving activity in the first hours after dawn (8:00 AM–10:00 AM) and dusk (8:00 PM–11:00 PM), respectively (pers. obser.). Chrysomelids were determined by Astrid Eben (Instituto de Ecología, Veracruz, Mexico).

Wild Lima bean belonging to the Mesoamerican gene pool grows abundantly in the study area (Heil, 2004). Young seedlings were collected from the same site and grown in 250 ml plastic cups filled with natural soil for at least 3 wk prior to the experiment. At the time of the experiment, plants were 40–60 cm tall, had 10 to 15 leaves, and were easily identifiable based on the typical trifoliate and extrafloral nectaries-bearing leaves (Heil, 2004). Plants were

sorted by pairs according to shoot length, leaf number, and average leaf size; then one plant per pair was selected randomly and induced between 08:00 and 09:00 AM, i.e., sprayed with 5 ml of a 1 mmol aqueous solution of JA (leaves were sprayed once until completely soaked). Control plants received equal amounts of water. Plants were allowed to dry, and then were put into PET bags ('Bratenschlauch', Toppits, Minden, Germany, a PET foil that does not emit detectable amounts of volatiles even after exposure to temperatures of up to 150°C), and placed so that they received a natural photoperiod without being exposed to direct sunlight.

Olfactometer Experiments. Tests on the beetles' behavior toward HIPV were conducted in a Y-olfactometer. Inflowing air was cleaned by charcoal filters (1.5 mg of charcoal, CLSA-Filters, Le Ruissaeu de Montbrun, France) and then passed plants placed in PET foil bags for 12 hr prior to the experiment. Air was kept flowing (ca 3l min^{-1}) by means of a ventilator at the end of the olfactometer. All experiments were conducted in the dark between 06:00 PM and 11:00 PM at a temperature of 28–30°C (air humidity >90%). Directional movement of the beetles was achieved by one lamp (40 W) placed 50 cm in front of the olfactometer. Each beetle was tested individually, and only beetles entering one of the arms within 5 min were counted as having made a choice. The arms of the olfactometer were exchanged after every fifth beetle. Each experiment consisted of one choice situation (control vs. induced plant, [C:I]) and three controls (empty arm vs. empty arm [0:0], empty arm vs. control plant [0:C], and control plant vs. control plant [C:C]). The experimental setup was conducted twice on two consecutive days, each with different plant pairs and different sets of beetles for the Chrysomelids, and with the same two plants pairs yet different sets of beetles for the Curculionids.

Feeding Experiments. Single beetles were placed in 250 ml plastic cups, each containing one leaflet of an induced plant and one of a control plant. Only lateral leaflets were used for this experiment, and only beetles that fed within 24 hr were evaluated. Leaflets were scanned to calculate missing leaf area using standard image-processing software, and the two leaflets exposed to the same beetle were used as a pair for data evaluation.

Gas Chromatography-Mass Spectrometry. Treated and control plants were generally tested for successful induction by using a portable GC (ZNose® Model 4100 Vapor Analysis Systems, Newbury Park, CA 91320). However, Curculionid beetles responded differently to odors of two induced plants, and volatiles released by these plants were collected after the olfactometer experiments over the next 24 hr on charcoal filters in a closed-flow stripping, resolved in dichloromethane to which *n*-bromodecane (200 ng μl^{-1}) had been added as internal standard (IS), and then subjected to GC-Trace-MS (2000 series, Thermo Quest, program Xcalibur 1.2, Finnigan Corp.; see Koch et al., 1999 for details and Hopke et al., 1994 for original identification of compounds).

RESULTS AND DISCUSSION

Whereas only traces of *cis*-ß-ocimene, C_{11} homoterpene, and $C_{10}H_{14}$ (compounds 1, 3, and 5 in Figure 1) and MeSA were released by control plants, JA treatment of wild Lima bean significantly induced release of several HIPV (data not shown, but see Figure 2 in Heil, 2004 for typical chromatograms of induced and control plants). Both Chrysomelids significantly preferred HIPV of controls to those of induced plants ($P < 0.01$ for both species on two days each, Figure 1). The beetles never showed a significant choice when offered two empty arms or two arms containing control plants, and they significantly preferred control plants vs. an empty arm (Figure 1). Therefore, these beetles' behavior represents an ecologically relevant avoidance of HIPV released by induced plants, at least when non-induced plants are also available. In the feeding experiments, these species preferred control leaflets ($P < 0.05$ in two independent feeding experiments conducted for each species, see Table 1).

Curculionid beetles significantly preferred the induced plant of one pair and did not choose in the case of the second pair (Figure 1). This pattern was repeated using the same plants yet different beetles one day later. HIPV released by these induced plants differed strongly from each other (Figure 1): Plant 1 was slightly induced and released only few HIPV at amounts lower than the IS. Plant 2, which was strongly induced, released *trans*-ß-ocimene, *cis*-jasmone, ß-caryophyllene, and TMTT at rates as high as or higher than the IS, and several other HIPV at lower rates (Figure 1). HIPV released in response to intense weevil feeding are usually dominated by trans-ß-ocimene, *cis*-jasmone, and TMTT (unpublished data obtained with ZNose), and all these compounds were released in high amounts by plant 2. In feeding experiments (same plant individuals), the Curculionids preferred the control plant to the induced one of pair 2 (heavily induced plant; $P = 0.057$) but showed no detectable preference in the case of pair 1 (slightly

TABLE 1. RESULTS OF FOOD CHOICE TESTS ON JA-INDUCED AND CONTROL LEAFLETS OF LIMA BEAN

	Cerotoma		*Gynandrobrotica*		Curculionidae	
	C:I	**P**	**C:I**	**P**	**C:I**	**P**
Trial 1	7:0	0.008	7:0	0.008	9:7	n.s.[1]
Trial 2	6:1	0.014	12:2	0.011	12:6	0.057[2]

C:I = number of control leaflets damaged: number of induced leaflets damaged, P = result of Wilcoxon test for paired samples on leaf area removed. Different trials were conducted with different plant pairs on different days.

[1]Plant pair 1, [2]Plant pair 2 (see Figure 1).

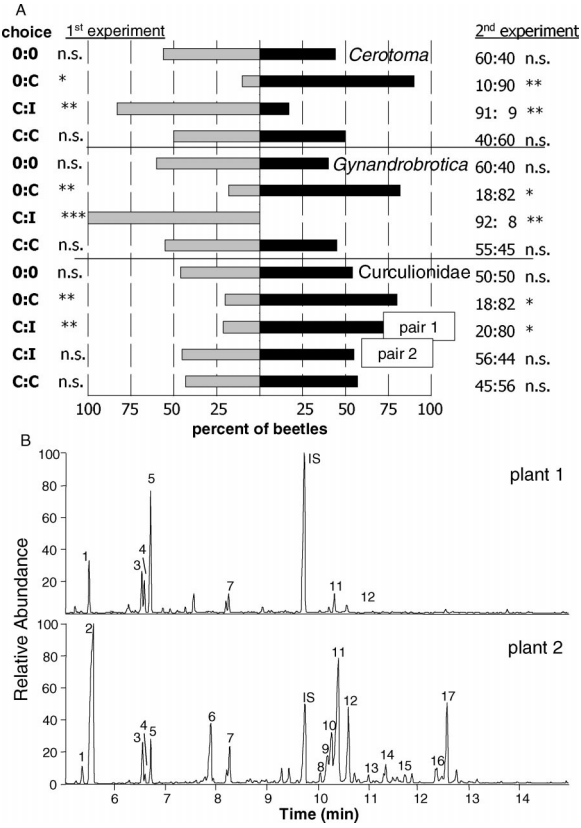

FIG. 1. Responses of beetles in different choice situations in a Y-tube olfactometer (A) and HIPV released by two different induced plants (B). (A) Behavior of three beetle species in different choice situations. Bars indicate percent of beetles that chose during the first experiment, trials (order from top to bottom indicates the temporal order of the experiment) were 0:0 (empty vs. empty), 0:C (empty vs. control), C:I (control vs. induced) and C:C (control vs. control). Results of tests against binomial distribution are indicated for both experiments (n.s. not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Data are graphically presented for the first experiment, while ratios are given for the second experiment. Sample sizes are: *Cerotoma*, 12 on day 1 and 15 on day 2; *Gynandrobrotica*, 10 on day 1 and 11 on day 2; Chrysomelidae, 16 on day 1 and 20 on day 2. (B) HIPV released by the induced plants of pairs 1 and 2 used in the experiment on weevils. Peaks are: 1: *cis*-ß-ocimene, 2: *trans*-ß-ocimene, 3: C_{11} homoterpene [4,8-dimethylnona-1,3,7-trien], 4: *p*-mentha-1,5,8-triene, 5: $C_{10}H_{14}$ [all-trans-2,6-dimethyloctatetraen], 6: *cis*-hexenylbutanoat, 7: $C_{10}H_{16}O$ [2,6-dimethyl-3,5-7-octatrien-2-ol], IS: *n*-bromodecane, 8: α-copaene, 9: *cis*-hexenylhexanoate(??), 10: hexenylbenzoate (??), 11: *cis*-jasmone, 12: ß-caryophyllene, 13: α-humulene, 14: germacrene, 15: α-farnesene, 16: nerolidol, 17: TMTT [(3E, 7E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene].

induced plant; see Table 1). These beetles feed on a slightly induced plant as they do on an uninduced one, and appear to use the HIPV of the slightly induced plant to locate it, yet they avoid heavily induced plants. Similar behavior has recently been reported for spider mites, which are also attracted to volatiles released by slightly induced Lima bean leaves, yet repelled by heavily induced leaves (Horiuchi et al., 2003). Releasing HIPV by slightly induced Lima beans, thus, can incur ecological costs by attracting, rather than deterring, herbivores.

Odors of control plants were more attractive than those of induced plants in all cases in which beetles preferred controls. Although beetles tend to be attracted to HIPV (pers. comm. by T. Turlings; see also Bolter et al., 1997; Kalberer et al., 2001), HIPV released by JA-induced wild Lima bean plants deterred both herbivorous Chrysomelid species and, thus, acted as a direct defense. In contrast, the Curculionids showed mixed responses. Why did conspecific plants respond differently to the same treatment? Are other herbivores also attracted by slightly induced plants yet deterred when HIPV release exceeds a given level? Further studies are required to determine if HIPV released by induced Lima beans under natural conditions act as a direct defense by deterring herbivores or if, instead, they attract herbivores, thus incurring ecological costs.

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SEX PHEROMONE OF QUEENS OF THE SLAVE-MAKING ANT, *Polyergus breviceps*

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Abstract—The sex attractant pheromone produced in mandibular glands of queens of the slave-making ant *Polyergus breviceps* has been identified as a blend of methyl 6-methylsalicylate and 3-ethyl-4-methylpentanol. In field trials, each compound alone was completely unattractive to males, whereas blends of the two compounds attracted hundreds of males within a couple of hours.

Key Words—*Polyergus breviceps*, queen sex pheromone, methyl 6-methylsalicylate, 3-ethyl-4-methylpentanol, ant, Formicidae.

INTRODUCTION

The organization of colonies of social insects such as ants (Formicidae) that are made up of thousands of individuals has fascinated mankind for millennia. Within the Formicidae, a small subset of species, the slave-making ants, are obligate social parasites that depend entirely on workers of slave species to carry out the tasks that enable a colony to survive (Topoff, 1999). Here we report the identification of the sex pheromone blend produced by virgin queens of *Polyergus breviceps*, a slave-making species that inhabits the western United States, where it exclusively parasitizes ants in the genus *Formica* (Topoff, 1999). Slave raids are conducted in early summer around the onset of summer rains, with raiding columns and winged males emerging from nests in mid- to late afternoon. Virgin queens accompany workers in the raiding columns, and during these raids, a queen releases a pheromone that immediately attracts swarms of flying males, one of which quickly mates with her. The newly mated *Polyergus* queen invades

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and takes over a colony of a *Formica* species, killing the resident *Formica* queen (Topoff, 1999). The subjugated *Formica* workers then rear her brood as their own. Subsequently, the new *Polyergus* workers, accompanied by their *Formica* slaves, raid nests of the slave species, returning with worker pupae that become new slave workers as they emerge as adults in the mixed colony. Remarkably, this appears to be only the second queen sex pheromone to be fully identified from ants, from the more than 10,000 described ant species (Ayasse et al., 2001).

METHODS AND MATERIALS

Field Site. Insects were collected and field bioassays were conducted at the Southwestern Research Station of the American Museum of Natural History in Portal, AZ located in the Chiricahua Mountains (altitude 1646 m) in an oak-juniper-pinyon pine woodlands.

Preparation and Analysis of Extracts. Insects were collected from raiding columns by aspiration, immediately killed by freezing, and cut into head, thorax, and abdomen. Frozen body parts in 1.5 ml screwcap glass vials were shipped on dry ice to UC Riverside for analysis. Solvent extracts were prepared by soaking body parts in $\sim$200 μl methylene chloride for $\sim$1 hr. Solid phase microextraction (SPME) collections were made by mashing thawed body parts in the vials with a glass rod, sealing the vial with foil, pushing the SPME needle through the foil, and exposing the SPME fiber (100 micron PDMS coating; Supelco, Bellefonte, PA) in the headspace for 30 min. The loaded fibers were desorbed in the GC-MS injector for 30 sec at 250°C. GC-MS analyses of SPME and solvent extracts were carried out with an Hewlett-Packard 6890 GC with liquid CO_2 cryogenic cooling, equipped with a 30 m $\times$ 0.25 mm HP-5MS column, interfaced to an HP 5973 mass selective detector, program 0°/1 min, 10°/min to 250°C. Coupled gas chromatography-electroantennogram detection (GC-EAD) analyses were conducted with heads of males, with the tip of one antenna inserted into the saline-filled glass capillary recording electrode, and the ground electrode inserted into the head. A DB-5 column was used for GC-EAD analyses of SPME-collected volatiles from squashed heads of queens, program 40°/1 min, 10°/min to 275°C. Methyl 6-methylsalicylate (Seidel et al., 1990) and 3-ethyl-4-methylpentanol standards (Bühring et al., 1976) were synthesized as previously described. Authentic standards of the other compounds listed in Table 1 were purchased or synthesized as required.

Field Bioassays. Body parts squashed on filter papers were placed in the center of Pherocon® AM sticky traps placed on the ground, about 3 m apart, and at least 15 m from the nearest nest. Traps, replicated seven times over several days, were deployed around $\sim$15:00 hr and recovered $\sim$2 hr later. Synthetic chemicals were bioassayed as hexane solutions loaded onto 11 mm grey rubber septa, which were placed in sticky traps deployed as described above. Treatments were

TABLE 1. COMPOUNDS IDENTIFIED FROM SOLID PHASE MICRO-EXTRACTION AND GC-MS ANALYSIS OF VOLATILES FROM SQUASHED HEADS OF DIFFERENT CASTES OF *Polyergus breviceps*[a]

	Relative amounts		
Compound	Queen	Worker	Male
Nonane*	+	++	
3,4-dimethylpentanol	++	++	++
3-ethyl-4-methylpentanol	++	+++	++++
unknown methyl ester	++	++	+++
octanol*	++		
m-cresol	+		
undecane*	+	++++	
3-ethyl-4-methylpentyl acetate	+	+	++
decanol*	++		
tridecane*		+	
methyl 6-methylsalicylate*	++++	+	+++
octyl butyrate*	++		++
ethyl 6-methylsalicylate	+		+++
decyl acetate*	++	+	
geranyl acetone*	+		++
2-tridecanone*	+	+	
decyl butyrate*	++		

[a] Because SPME is a qualitative collection method, with amounts of compounds collected dependent on their volatilities, the amounts of compounds collected were categorized as + (GC peak < 1% of major component), ++ (1–25% of major component), +++ (26–60% of major component, ++++ (major component). Heads from 12 queens, 4 workers, and 6 males were sampled.

randomized within a replicate, and replicates were spaced ~50 m apart. Traps were left on the ground for ~2 hr, then retrieved for counting. In the first bioassay ($N = 5$), septa were loaded with 1000 μg of the blend, whereas in the second bioassay ($N = 10$), 100 μg doses were used to obtain better discrimination between blends. Differences between means were determined by Newman-Keuls tests run on the square-root transformed data ($\alpha = 0.05$).

RESULTS AND DISCUSSION

In previous studies testing the attractiveness of squashed body sections, males were attracted to squashed heads from virgin queens, and subsequently, the mandibular glands were determined to be the source of the sex pheromone (Topoff and Greenberg, 1988). These bioassays were repeated, and as expected, odors from squashed queen heads attracted males, whereas odors from thoraxes or abdomens were minimally attractive. In total, traps baited with squashed heads caught 273 males, versus 2 and 8 males caught in traps baited with squashed thoraxes and

abdomens respectively ($N = 7$). The attraction persisted for less than one hr, suggesting that the pheromone was either quite volatile or relatively unstable.

Methyl 6-methylsalicylate was readily identified by gas chromatography-mass spectrometry (GC-MS) as the only volatile compound in preliminary CH_2Cl_2 extracts of the heads or dissected mandibular glands of virgin queens that had been stored in a freezer for several months. However, this compound was completely unattractive to males in field trials in 2001. A more thorough investigation and comparison of the volatiles from squashed heads of freshly collected virgin queens, workers, and males conducted in 2002, using solid phase microextraction (SPME) (Pawliszyn, 1997), produced several important results. First, a number of components were identified from squashed heads of all three castes (Table 1), and although there were marked differences in the relative proportions of the compounds found in each caste, there were few compounds that appeared to be caste specific. Second, the amount of material recovered by SPME collections from queen heads was at least an order of magnitude more than that recovered from heads of the other two castes (e.g., relative amounts of methyl 6-methylsalicylate in SPME extracts of squashed heads of queens, workers, and males was 100: 0.08: 0.2, respectively).

The identification of all the compounds listed in Table 1 was not completed in time for field-testing in 2002, but a partially reconstructed queen blend, and the individual components of the blend (asterisked in Table 1), were tested, with completely negative results. Following these setbacks, we analysed volatiles collected from queen heads by SPME with GC-EAD. Antennae of males responded strongly to two compounds in the extracts, including the previously identified methyl 6-methylsalicylate and another earlier eluting compound (Figure 1). Traces of a third compound, eluting after methyl 6-methylsalicylate, elicited weaker antennal responses. This compound was identified as the ester analog, ethyl 6-methylsalicylate, and it seems likely that the antennal responses to this compound were artefactual, given its trace quantity and its close structural similarity to methyl 6-methylsalicylate.

The early-eluting compound that stimulated strong antennal responses from antennae of males was identified as 3-ethyl-4-methylpentanol from its chromatographic and mass spectral characteristics, including a tentative match with a published spectrum (Francke et al., 1985). The identification was confirmed by synthesis of an authentic standard. Analyses of concentrated CH_2Cl_2 extracts of mandibular glands of freshly collected queens determined that methyl 6-methylsalicylate (1608 ± 310 ng/queen, $N = 4$) and 3-ethyl-4-methylpentanol (184 ± 39 ng/queen) comprised more than 99% of the extractable volatile material in the glands.

Field trials in 2003 with blends of these two compounds produced immediate and dramatic results. In the first trial, using total doses of 1000 μg, traps baited with the individual components caught no males, whereas traps baited with blends

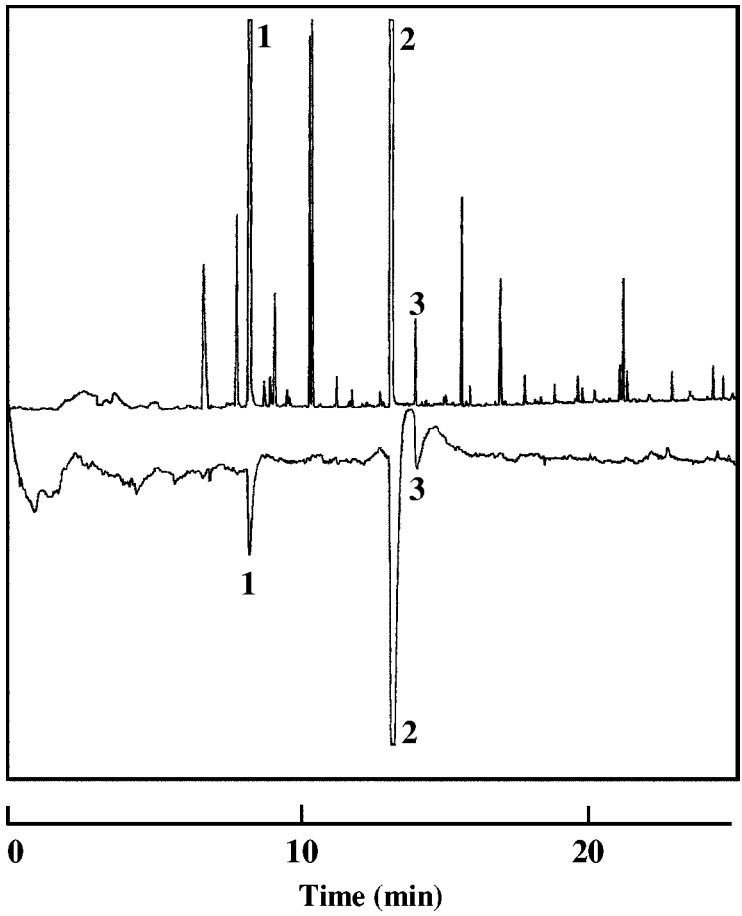

FIG. 1. Coupled gas chromatogram-electroantennogram showing responses by an antenna of a male *Polyergus* to volatiles from the squashed head of a virgin queen. Upper trace is the chromatogram, lower inverted trace is the antennal response. Peak 1: 3-ethyl-4-methylpentanol, Peak 2: methyl 6-methylsalicylate, and Peak 3: ethyl 6-methylsalicylate. GC analysis conditions: DB-5, 30 m $\times$ 0.25 mm id $\times$ 0.25 μm film. Temperature program: 40°C/1 min, 10°/min to 275°C.

attracted hundreds of males over a two hr period (alcohol: salicylate ratio 4:1, 32.2 $\pm$ 10.3 males [mean $\pm$ SE, $N = 5$]; 1:1, 174.8 $\pm$ 31.6 males; 1:4, 154.4 $\pm$ 34.6 males). The latter two treatments were equivalent, whereas the 4:1 ratio was less attractive (Newman-Keuls test). A second trial ($N = 10$), using 100 μg total doses and a narrower range of blends, verified the biological activity of the blends, with a 9:1 ratio (1.3 $\pm$ 0.34 males) of alcohol to salicylate being less attractive

than the statistically equivalent 3:1 (16.4 ± 4.0 males) and 1:9 ratios (9.4 ± 1.6 males). These ratios in turn were less attractive than the 1:1 (40.8 ± 4.8 males) and 1:3 ratios (47.2 ± 9.6 males), which were equal in activity. Thus, in both trials, blends consisting of 50–80% salicylate were most attractive to males. These results, coupled with the electroantennogram and analytical results, confirm that the queen's sex attractant pheromone consists of methyl 6-methylsalicylate and 3-ethyl-4-methylpentanol, and that both components are essential for biological activity. The volatility of 3-ethyl-4-methylpentanol also suggests why the attractiveness of squashed queen heads dissipates quickly. The absolute configuration of the insect-produced 3-ethyl-4-methylpentanol has not yet been determined, but the strong attraction of males to blends containing the racemic material suggests that the "incorrect" enantiomer is not inhibitory.

The site of production and chemistry of the pheromone are different in every respect from that of *Formica lugubris* (Walter et al., 1993), the only other ant species for which a queen sex pheromone has been fully identified. Specifically, in the latter species, the pheromone is produced in the Dufour's gland of the queen as opposed to the mandibular glands, and the pheromone consists of the straight-chain hydrocarbons undecane, tridecane, and (*Z*)-4-tridecene, in contrast to the branched chain alcohol and salicylate components of the *P. breviceps* pheromone.

Methyl 6-methylsalicylate has been previously reported from several ant species, in which it serves a number of diverse roles, including as a pheromone triggering the flight of *Camponotus* sexuals from the nest (Ayasse et al., 2001). It also was recently reported as the sole volatile constituent in mandibular glands of *Polyergus rufipes*, a European congener of *P. breviceps*, but its biological role in that species has not been verified (Castracani et al., 2003). The second pheromone component, 3-ethyl-4-methylpentanol, has been identified from mandibular glands of workers of *Harpegnathos saltator* (Nascimento et al., 1993) and in heads of *Formica rufa* workers and *Formica polyctena* queens (Francke et al., 1985). However, the biological relevance of this compound for these three species has not been reported. The fact that both of the pheromone components of *P. breviceps* have been found in other ant species, coupled with the fact that a blend of components was essential in obtaining behavioral responses, suggests that reconstruction of blends of volatiles released by queen ants of other species, in tandem with GC-EAD analyses, may result in the rapid elucidation of queen sex pheromones for other ant species.

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EXPOSURE OF LIMA BEAN LEAVES TO VOLATILES FROM HERBIVORE-INDUCED CONSPECIFIC PLANTS RESULTS IN EMISSION OF CARNIVORE ATTRACTANTS: ACTIVE OR PASSIVE PROCESS?

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Abstract—There is increasing evidence that volatiles emitted by herbivore-damaged plants can cause responses in downwind undamaged neighboring plants, such as the attraction of carnivorous enemies of herbivores. One of the open questions is whether this involves an active (production of volatiles) or passive (adsorption of volatiles) response of the uninfested downwind plant. This issue is addressed in the present study. Uninfested lima bean leaves that were exposed to volatiles from conspecific leaves infested with the spider mite *Tetranychus urticae*, emitted very similar blends of volatiles to those emitted from infested leaves themselves. Treating leaves with a protein-synthesis inhibitor prior to infesting them with spider mites completely suppressed the production of herbivore-induced volatiles in the infested leaves. Conversely, inhibitor treatment to uninfested leaves prior to exposure to volatiles from infested leaves did not affect the emission of volatiles from the exposed, uninfested leaves. This evidence supports the hypothesis that response of the exposed downwind plant is passive. *T. urticae*-infested leaves that had been previously exposed to

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volatiles from infested leaves emitted more herbivore-induced volatiles than *T. urticae*-infested leaves previously exposed to volatiles from uninfested leaves. The former leaves were also more attractive to the predatory mite, *Phytoseiulus persimilis*, than the latter. This shows that previous exposure of plants to volatiles from herbivore-infested neighbors results in a stronger response of plants in terms of predator attraction when herbivores damage the plant. This supports the hypothesis that the downwind uninfested plant is actively involved. Both adsorption and production of volatiles can mediate the attraction of carnivorous mites to plants that have been exposed to volatiles from infested neighbors.

Key Words—*Phytoseiulus persimilis*, *Tetranychus urticae*, lima bean, herbivore-induced plant volatiles, plant–plant interactions.

INTRODUCTION

Ecosystems are composed of complex food webs consisting of trophic interactions between plants and animals and among animals (Polis and Strong, 1996). In addition to direct trophic interactions, there are many indirect interactions (Abrams et al., 1996). For example, plants may interact with carnivorous animals through plant volatiles whose production is induced by herbivory; these volatiles are called herbivore-induced plant volatiles (hereafter called HIPV) (Vet and Dicke, 1992; Turlings et al., 1995; Takabayashi and Dicke, 1996; Dicke et al., 2003). When these volatiles attract carnivorous enemies of herbivores, and consequently reduce the herbivores' damage, the release of volatiles by plants is considered to be an induced, indirect defense (Price et al., 1980; Dicke and Sabelis, 1988; Turlings et al., 1993; Takabayashi and Dicke, 1996; Dicke, 1999; Sabelis et al., 1999). The attraction of carnivorous arthropods can enhance the fitness of plants that are infested with herbivorous arthropods (Van Loon et al., 2000; Hoballah-Fritzsche and Turlings, 2001).

A well documented system with induced, indirect plant defense consists of lima bean plants, two-spotted spider mites (*Tetranychus urticae*), and natural enemies of the spider mites. When spider mites infest lima bean leaves, the leaves emit feeding-induced volatiles that attract carnivores, such as the predatory mites *Phytoseiulus persimilis* (Dicke et al., 1999) and *Amblyseius womersleyi* (Maeda et al., 1999), and the predatory insects *Scolothrips takahasii* (Shimoda et al., 1997, 2002) and *Oligota kashmirika benefica* (Shimoda and Takabayashi, 2001; Shimoda et al., 2002). Once the infested plants emit HIPV, the volatiles can also be exploited by other organisms, such as conspecific and heterospecific herbivores (e.g., Dicke, 1986; Bernasconi et al., 1998; Landolt et al., 1999; Dicke and Van Loon, 2000; Horiuchi et al., 2002), and neighboring plants (see review in Dicke and Bruin, 2001).

In this paper, we focus on the effects of herbivore-induced plant volatiles on neighboring plants. In the tritrophic system of lima bean plants, *T. urticae* and

P. persimilis, Dicke et al. (1990) showed that uninfested lima bean plants that had been exposed to volatiles from *T. urticae*-infested lima bean plants were more attractive to *P. persimilis* than uninfested, unexposed plants. Two hypotheses might explain their results: (1) the emitted HIPV induce the production of carnivore-attracting volatiles in neighboring plants; or (2) HIPV *adsorbed* onto the neighboring plants, and were subsequently released, which resulted in predator attraction (Dicke et al., 1990; Bruin et al., 1995). The first hypothesis involves an active mechanism, while the second involves a passive mechanism. Recently, Arimura et al. (2000a,b, 2001) reported that defense genes were activated in uninfested lima bean plants in response to exposure to volatiles from *T. urticae*-infested lima bean plants. This shows that the exposed plants actively responded to the volatiles. However, it remains to be investigated whether such exposed, uninfested plants also actively produce predator-attracting volatiles.

In this study we addressed the effects of exposure of undamaged lima bean plants to HIPV on the emission of predator-attracting volatiles. We especially asked whether the exposed undamaged leaves are actively (production of volatiles) or passively (adsorption and re-release of volatiles) involved.

METHODS AND MATERIALS

Plants and Mites. Lima bean (*Phaseolus lunatus L.*) plants were grown in soil in a greenhouse at 25 ± 2°C, 60–70% relative humidity (RH), and a photoperiod of 16 hr. We used plants that were grown for 10–15 days after germination; these plants had two primary leaves, and the first trifoliate leaf had just started to unfold.

Herbivorous spider mites (*Tetranychus urticae* Koch) were reared on kidney bean plants (*Phaseolus vulgaris* L), grown under similar conditions to the lima bean plants, but in a climate-controlled room (25 ± 2°C, 60–70% RH, 16L-8D). Adult females were used for all experiments. Predatory mites (*Phytoseiulus persimilis* Athias-Henriot) were reared on detached kidney bean leaves that were heavily infested by spider mites, under the same conditions. New *T. urticae*-infested leaves were added every other day.

Chemical Analysis. Leaf volatiles were collected in 2 l glass containers. Volatile compounds were drawn from the headspace of the container by holding the infested plant in a glass tube packed with Tenax TA adsorbent (100 mg, mesh 20/35) for 3 hr, at a flow rate of 100 ml/min in a climate-controlled room at 25 ± 2°C.

The adsorbed compounds were eluted with 2 ml of diethyl ether, and 0.5 μg of *n*-eicosane (the internal standard) was then immediately added to the eluate. The eluate was concentrated with a stream of gaseous N_2 and injected into the injection port (250°C) of a gas chromatograph-mass spectrometer (GC-MS) [GC: Hewlett Packard 6890 with an HP-5MS capillary column (i.d. = 0.25 mm, length = 30 m,

film thickness = 0.25 μm); MS: Hewlett Packard 5973 mass selective detector, 70 eV]. The GC oven temperature was programmed to rise from 40°C (5-min hold) to 280°C at 15°C/min.

The chemical structure of each compound was elucidated by comparing the mass spectra and retention time with those of authentic chemical samples. (*E*)-β-ocimene, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) were provided by W. Boland (Max Planck Institute of Chemical Ecology, Germany). Linalool and methyl salicylate (MeSA) were purchased from Wako Chemical Company, Japan. Peak area of each compound in the total ion currency was measured by relative comparison with the internal standard. Emission rates were statistically compared between treatments for each compound by using the Mann-Whitney *U* test.

Primary leaves of lima bean plants were used in all experiments by cutting the petiole of a leaf from the plant with a razor blade. Individual petioles, with an attached leaf, were inserted into a 6 ml vial with either the test solution or distilled water as control. One leaf of each plant was assigned to the treatment and the other to the control to reduce variation between treatments ($N = 5$).

Volatiles from Uninfested Lima Bean Leaves That Had Been Exposed to Volatiles from T. urticae*-Infested Conspecific Leaves.* The petiole of uninfested, detached leaves was inserted into a 6-ml vial with either an aqueous solution of the protein synthesis inhibitor cycloheximide (10 μg/ml) or distilled water. After 3 hr, 300 *T. urticae* females were placed on each of five uninfested, water-treated leaves or uninfested, cycloheximide-treated leaves. After 1 day, the volatiles emitted from these leaves were collected and analyzed with GC-MS.

To confirm that water-treated leaves and cycloheximide-treated leaves were equally infested by *T. urticae*, we counted the number of *T. urticae* eggs on each leaf. This was done in a separate experiment as follows. We prepared cycloheximide-treated and water-treated leaves as described above ($N = 29$). After 3 hr, we made a 1 × 1-cm-square barrier with a thin tanglefoot line on the surface of a leaf, and put one female in the square. After 24 hr, we measured the number of eggs in the square. The mean number of eggs was not significantly different between cycloheximide-treated and water-treated leaves (cycloheximide: 11.6 ± SE = 0.60, water: 10.3 ± 0.78; independent *t*-test $P = 0.187$). As the number of eggs is proportional to the amount of feeding (S. Yano, personal communication), the result shows that the cycloheximide-treatment did not significantly affect the feeding of *T. urticae* females.

Two water-treated leaves, each infested with 300 *T. urticae* females for 1 day, and three unifested water-treated leaves and three uninfested cycloheximide-treated leaves were introduced into an airtight 7-l glass container. The container was placed into a climate-controlled room at 25 ± 2°C and with a photoperiod of 16 hr. Care was taken to ensure that the spider mites on the infested leaves did not invade the uninfested leaves (see Arimura et al., 2000a for the details of the

experimental design). Under these conditions, uninfested leaves were exposed to volatiles from the infested leaves for 1 day (HIPV-exposed leaves). In the control experiment, two uninfested water-treated leaves were used instead of the infested leaves in the above setup. The exposed leaves were named "ULV-exposed leaves," where "ULV" stands for "uninfested leaf volatiles." Volatile compounds emitted by the exposed leaves were collected and analyzed with GC-MS, as described above.

Volatiles Emitted from Odor-Exposed Leaves That Were Subsequently Infested by T. urticae. In this experiment, we investigated the effect of previous exposure to HIPV on the emission of volatiles and attraction of predators in response to subsequent spider mite infestation. All the leaves used had their petioles in distilled water. Two infested leaves (300 female spider mites per leaf, 1-day post-infestation—odor source) and five uninfested leaves were introduced into an airtight 7-l glass container for 1 day. For the control experiment, two uninfested leaves were used as the odor source. Volatiles from exposed leaves were collected immediately after the exposure, as well as 1 or 3 days after the exposure, and then analyzed with GC-MS.

We prepared five leaves exposed to volatiles from *T. urticae*-infested leaves (HIPV-exposed leaves) and five leaves exposed to volatiles from uninfested leaves for 1 day (ULV-exposed leaves). Subsequently, 50 *T. urticae* females were placed onto each odor-exposed leaf. Volatile compounds released from the infested leaves were collected 1 and 3 days after the introduction of *T. urticae* and analyzed with GC-MS.

Two predatory mite "choice" experiments were conducted in a Y-tube olfactometer. Individual *Phytoseiulus persimilis* mites were offered a choice between (a) uninfested leaves vs. HIPV-exposed leaves that were subsequently infested by 80 *T. urticae* females for 1 day, or (b) uninfested leaves vs. ULV-exposed leaves that were subsequently infested by 80 *T. urticae* females for 1 day. In the center of the Y-tube olfactometer, was an iron, Y-shaped wire (for details of the olfactometer setup, see Takabayashi and Dicke, 1992). Leaf volatiles, in air cleaned with activated charcoal, were pushed into each of the arms of the olfactometer at a flow rate of 2.5 l/min. Adult female predatory mites that had been starved for 1 day were individually positioned at the beginning of the iron wire. When the predator reached the end of one of the arms of the olfactometer, the choice was recorded. The maximum time that each individual was allocated for reaching the end of an arm was 5 min. After every five mites, the alternate odor sources were swapped to the other arm to adjust for potential asymmetries in the experimental arena. Individual predators were used only once, and a total of 30 predators were used in 1 day. Each experiment was replicated on at least three different days, each with different odor sources and predatory mites. The test was performed in a climate-controlled room at $25 \pm 2^{\circ}$C and $70 \pm 10\%$ RH. The distribution of the predators over two odor sources was analyzed with a binomial test to determine whether the distribution was significantly different from a 1:1 distribution. Fisher's

exact probability test was used to determine the significant differences between the distributions of predators over the two odor sources for different experiments. All experiments were done from October 2000 to August 2001.

RESULTS

Volatiles from Uninfested Lima Bean Leaves That Had Been Exposed to Volatiles from T. urticae*-Infested Conspecific Leaves.* Infested water-treated leaves released more (*E*)-β-ocimene, linalool, DMNT, MeSA, and TMTT than infested cycloheximide-treated leaves, which emitted only trace amounts of these volatiles (Figure 1a). Conversely, both HIPV-exposed cycloheximide-treated leaves and

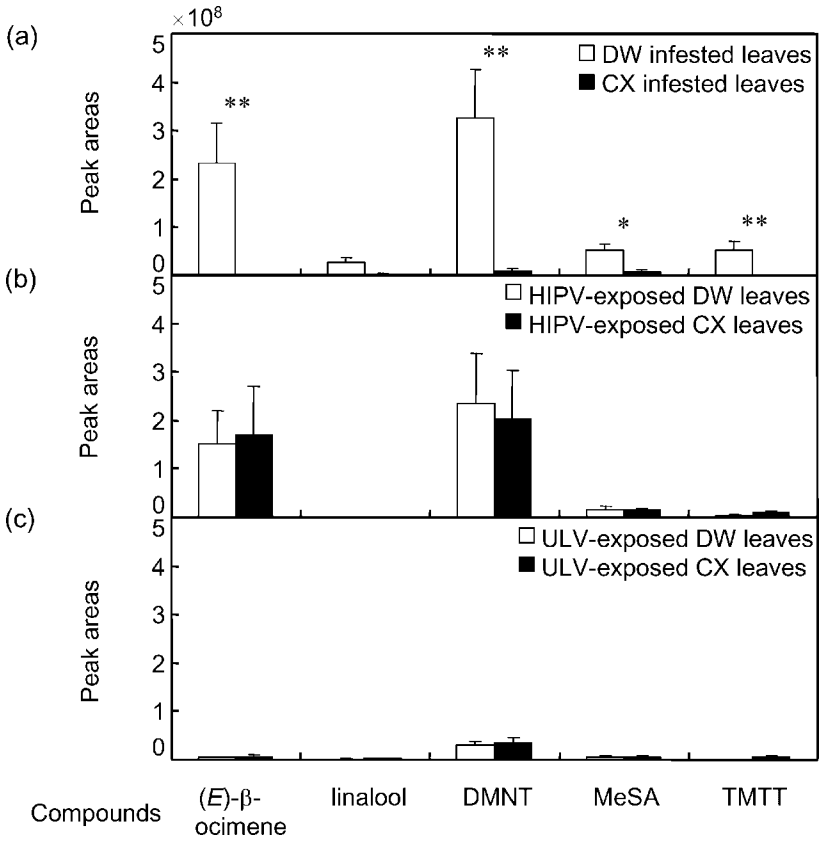

FIG. 1. The effect of cycloheximide on the production of volatiles by (a) *T. urticae*-infested leaves, (b) HIPV-exposed leaves, and (c) ULV-exposed leaves (ion intensities per leaf; mean ± SE). $^{*} = P < 0.05$, $^{**} = P < 0.01$.

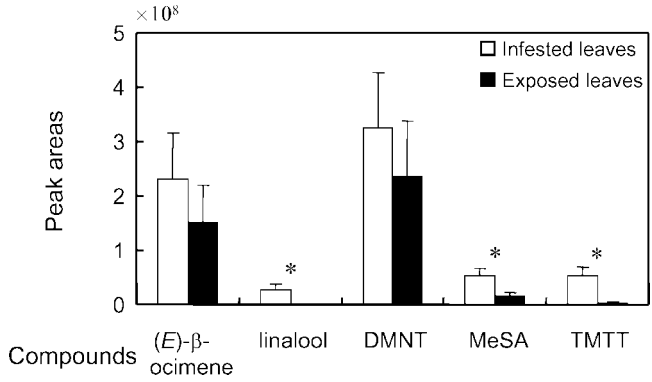

FIG. 2. Comparison of volatile emission rates from spider-mite infested leaves and leaves exposed to odors from spider-mite infested leaves (ion intensities per leaf; mean ± SE). * = $P < 0.05$.

HIPV-exposed water-treated leaves released large amounts of these volatiles, and the amounts were not significantly different among treatments for any of the compounds (Figure 1b). Trace amounts of (E)-β-ocimene, DMNT, and MeSA were recorded in ULV-exposed water-treated leaves and ULV-exposed cycloheximide-treated leaves, and trace amounts of TMTT were recorded in only ULV-exposed cycloheximide-treated leaves (Figure 1c). Infested water-treated leaves emitted significantly more linalool, MeSA, and TMTT than HIPV-exposed water-treated leaves. (Figure 2).

Volatiles Emitted from Odor-Exposed Leaves That Were Subsequently Infested by T. urticae. All leaves used in the experiments had their petioles in distilled water. HIPV-exposed uninfested leaves released more (E)-β-ocimene, DMNT, and MeSA than ULV-exposed uninfested leaves just after the exposure (Figure 3). However, a rapid reduction in the amounts of these compounds emitted from HIPV-exposed uninfested leaves, except for MeSA, was observed 1 and 3 days after exposure (Figure 3).

One day after the introduction of *T. urticae*, HIPV-exposed leaves emitted more (E)-β-ocimene and DMNT than ULV-exposed leaves (Figure 4a). HIPV-exposed leaves still released larger amounts of those volatiles than ULV-exposed leaves 3 days after the introduction of *T. urticae*, but the differences were not statistically significant (Figure 4b).

More predators were attracted to HIPV-exposed infested leaves than uninfested leaves (Figure 5b), whereas the predators were equally distributed between ULV-exposed infested leaves and uninfested leaves (Figure 5a). The results of these two olfactometer experiments are significantly different (Fisher's exact probability test, $P = 0.007$).

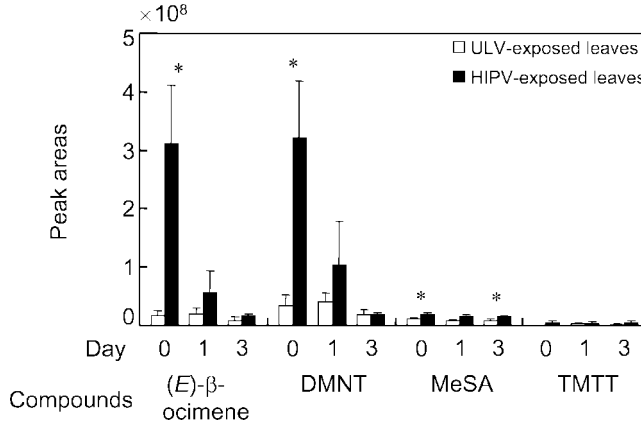

FIG. 3. Emission rates of volatiles emitted from odor-exposed leaves at 0, 1, and 3 days after exposure (ion intensities per leaf; mean ± SE). * = $P < 0.05$.

DISCUSSION

As pointed out by Dicke et al. (1990) and Bruin et al. (1995), there are two hypotheses to explain the volatile emissions from leaves that have been exposed to volatiles from infested leaves: (1) HIPV are detected by exposed leaves, which results in the production of volatiles (the production hypothesis), and (2) HIPV are adsorbed onto the surface of exposed leaves and subsequently re-emitted (the adsorption hypothesis). Since cycloheximide-treatment resulted in the suppression of the induced volatiles, lima bean leaves do not use preformed enzymes together with the relevant substrates, but require protein synthesis for the production of herbivore-induced volatiles. Moreover, cycloheximide-application did not affect the emission of volatiles from HIPV-exposed leaves. These data support the adsorption hypothesis. If adsorption and re-release were the only cause of the volatile emissions from HIPV-exposed leaves, the blend of volatiles when these leaves were treated with cycloheximide, would be expected to be similar to that of the volatiles from infested leaves. However, a difference in blend composition of volatiles from HIPV-exposed leaves and infested leaves was observed: *T. urticae*-infested leaves emitted linalool, but HIPV-exposed leaves did not. Different adsorption, or re-release, rates for different compounds may explain this difference.

It is important to note that the production and adsorption hypotheses are not mutually exclusive. HIPV-exposed leaves that were subsequently infested by spider mites (*T. urticae*) for 1 day released significantly larger amounts of volatiles than the ULV-exposed leaves with the same level of infestation. The difference in volatile emissions cannot be explained by higher emission rates from HIPV-exposed,

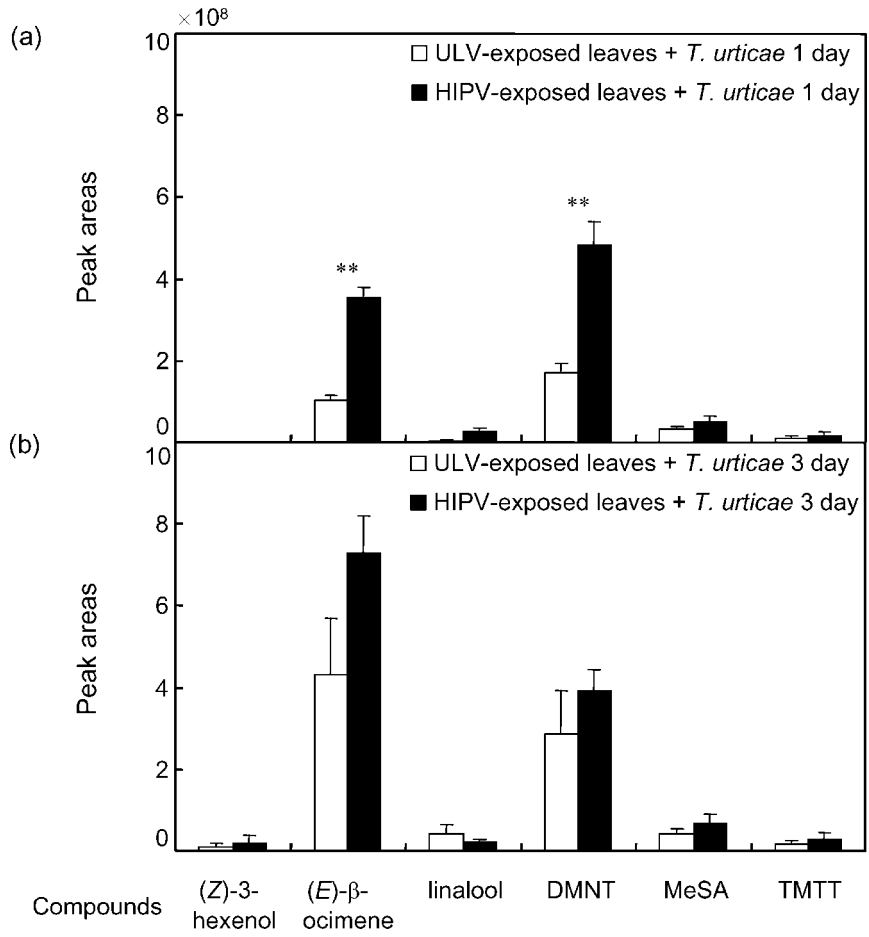

FIG. 4. Effects of odor exposure on the production of *T. urticae*-induced volatiles: (a) volatiles emitted from the exposed leaves one day after infestation and (b) volatiles emitted from the exposed leaves three days after infestation (ion intensities per leaf; mean ± SE). ** = $P < 0.01$.

uninfested leaves than from ULV-exposed, uninfested leaves, because the volatiles from HIPV-exposed leaves and ULV-exposed leaves were not different 1 day after exposure (Figure 3), and the difference of (E)-β-ocimene and DMNT release between infested ULV-and HIPV-exposed leaves at day 1 (Figure 4a) was significantly higher (Mann-Whitney U test: $P < 0.05$) than that between ULV- and HIPV-exposed leaves (Figure 3). Therefore, these data indicate that changes related to the biosynthesis of volatiles occurred in the HIPV-exposed leaves, which

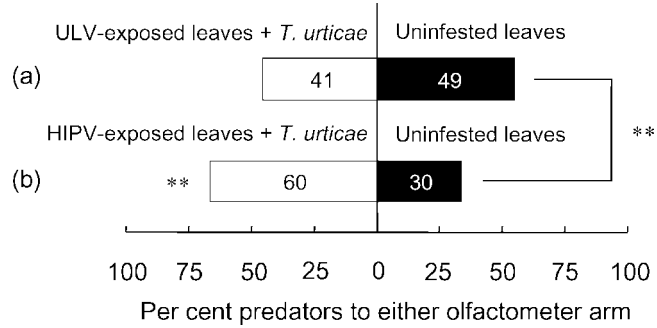

FIG. 5. The olfactory response of *P. persimilis* females to volatiles from odor-exposed leaves of different treatments, as determined in a Y-tube olfactometer: (a) uninfested leaves vs. HIPV-exposed water-treated leaves that were subsequently infested by 80 *T. urticae* females for 1 day, and (b) uninfested leaves vs. ULV-exposed water-treated leaves that were subsequently infested by 80 *T. urticae* females for 1 day. Asterisks besides the bar of choice (b) mean the significant difference between uninfested leaves and infested ULV-exposed leaves. Asterisks between the bars mean the significant difference in preference of predators between choice (a) and (b). ** $= P < 0.01$, Numbers in the bar segments represent the actual numbers of predators.

supports the production hypothesis. Recently, Engelberth et al. (2004) have reported that corn seedlings previously exposed to green leaf volatiles (GLV) from neighboring plants produced more jasmonic acid and volatile sesquiterpenes in response to mechanical damage and induction with caterpillar regurgitant than seedlings not exposed to GLV. The olfactometer bioassays showed that the infested HIPV-exposed leaves were also more attractive to the predatory mites than infested ULV-exposed leaves. This indicates that HIPV-exposed uninfested leaves produce more of the predator attractants when infested by spider mites than ULV-exposed uninfested leaves that are subsequently infested by spider mites. However, whether adsorbed volatiles might also contribute to the attractiveness of predatory mites by infested HIPV-exposed leaves remains unanswered.

One disadvantage of induced defense by plants is that there is a time lag between infestation and the induction of the defense. Maeda and Takabayashi (2001) studied the time course of HIPV-emission in *T. urticae*-infested kidney bean plants and found that the plants started to emit HIPV after there were approximately 50 (female) spider mites per leaf. They suggested that the indirect defense of HIPV by kidney bean plants might be induced after an increase of spider mites. Our present results (Figures 4 and 5) show that the time lag between infestation and volatile emission can be reduced by previous exposure to volatiles from conspecific neighbors. Mattiacci et al. (2001) reported analogous effects in cabbage plants that had been previously infested by the herbivore *Pieris brassicae*; when undamaged

leaves of previously infested cabbage plants were re-infested by conspecifics for a short time, such leaves were 10 times more attractive to the parasitoid *Cotesia glomerata* than recently infested leaves from plants that had not previously been infested.

In this paper, we have presented data in support of two possible ways in which undamaged plants, which are exposed to volatiles from infested conspecifics, become more attractive to predatory mites: (1) uninfested exposed leaves may recruit predators before being damaged, most likely through a passive process involving adsorption of predator-attractants, and (2) exposed plants increase their higher volatile emission rate as soon as they become infested by the herbivore species that infested their neighbors, through an active process. The latter phenomenon is likely to be important to HIPV-exposed plants, as it allows them to induce an indirect defense more intensively or at a faster rate than when they had not been exposed to the volatiles of their neighbors. Thus, a disadvantage of inducible defenses, i.e., the time delay between infestation and induction of a defense, may be attenuated by responding to volatiles from herbivore-infested neighbors. Our data add to the growing literature on infochemical-mediated interactions between damaged and undamaged plants (Dicke and Bruin, 2001 and references therein).

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RESPONSES OF FEMALE ORANGE WHEAT BLOSSOM MIDGE, *Sitodiplosis mosellana*, TO WHEAT PANICLE VOLATILES

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Abstract—Air entrainment samples of volatiles from panicles of intact wheat, *Triticum aestivum*, cultivar 'Lynx' were collected at the ear emergence/early anthesis growth stage. In an olfactometer bioassay, both freshly cut panicles and an air entrainment sample were found to attract female orange wheat blossom midge adults, *Sitodiplosis mosellana*. Coupled gas chromatography-electroantennography (GC-EAG) analyses of panicle volatiles located six electrophysiologically active components. These were identified by coupled gas chromatography-mass spectrometry and coinjection with authentic standards, on polar and nonpolar GC columns, as acetophenone, (*Z*)-3-hexenyl acetate, 3-carene, 2-tridecanone, 2-ethyl-1-hexanol, and 1-octen-3-ol. Although none of these was active when presented individually at the levels present in the entrainment sample, acetophenone, (*Z*)-3-hexenyl acetate, and 3-carene were active in the olfactometer when presented at a higher dose of 100 ng on filter paper. However, the six-component blend and a blend of acetophenone, (*Z*)-3-hexenyl acetate, and 3-carene, in the same ratio and concentration as in a natural sample, was as attractive to female *S. mosellana* as the whole air entrainment sample.

Key Words—Semiochemical, kairomone, volatile, host-plant, wheat, *Sitodiplosis*, Cecidomyiidae, electroanntenogram, olfactometer.

INTRODUCTION

Orange wheat blossom midge, *Sitodiplosis mosellana* (Géhin) (Diptera: Cecidomyiidae), is a common, though sporadic, pest of wheat in the Northern Hemisphere. Recent outbreaks have occurred in Canada, Finland, China, Japan,

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and the UK (Oakley et al., 1998). Over-wintering pupae can remain dormant in the soil for up to 13 years (Barnes, 1956). Climatic conditions influence the emergence of adult midges from these pupae and hence infestation varies from year to year. In the UK, precipitation causing moist soil conditions at the end of May, followed by warm, still weathter in late May/early June can lead to serious midge outbreaks and subsequent crop losses. In an outbreak in 1993, crop losses were estimated to have exceeded £30 million (Oakley, 1994). The actual degree of damage is hard to predict, and to be effective, insecticide has to be applied very soon after midges are detected (Oakley et al., 1998).

S. mosellana is a cereal specialist, for which wheat is the most attractive crop for oviposition, but in the absence of a wheat crop at a suitable growth stage, midges will fly to crops of rye, triticale, or barley, or may complete their life cycle in weed grasses. Females oviposit on panicles at any stage from the onset of heading up to and including anthesis, i.e., growth stages 46–69 (Tottman and Broad, 1987; Ding and Lamb, 1999). Larval feeding on the developing seeds causes shriveling and presprouting damage and also facilitates secondary fungal attack by *Fusarium graminearium* and *Septoria nodorum* (Oakley, 1994). This affects both the yield and quality of grain harvested.

The sex pheromone of *S. mosellana* has already been identified by Gries et al., (2000) and is highly attractive to male midges. However, host-plant volatile kairomones that attract female insects have not been previously identified for any Cecidomyiid species. Field observations have shown that female *S. mosellana* fly upwind, often over considerable distances, to wheat crops (Oakley, 1994). This suggested that attraction could be mediated by volatile semiochemicals emitted from wheat plants at appropriate growth stages. In this study, the response of *S. mosellana* to wheat panicle volatiles was investigated by coupled gas chromatography-electroantennography (GC-EAG) and olfactometer studies.

METHODS AND MATERIALS

Insect Rearing. Soil samples were taken from sites with severe *S. mosellana* damage, transferred to seed trays (5-cm depth), and stored at 5°C. After at least 3 mo. vernalization, trays were moved to a controlled environment cabinet (22°C, 75%RH, 16:8 L:D) and watered to bring the midges out of diapause. Adult emergence occurred within 3 wk of the trays being moved into these warmer, moister conditions. Female insects used for electrophysiology and bioassays were likely to have been mated since they were obtained from mixed sex containers.

Chemicals. EAG and laboratory behavioral studies were done using authentic samples of acetophenone, 2-tridecanone, 2-ethyl-1-hexanol, and (+)-3-carene (all 99% purity, Aldrich Chem. Co., Milwaukee, USA), and (*Z*)-3-hexenyl acetate and racemic 1-octen-3-ol (99% and 98% purity respectively, Avocado Research Chemicals Ltd., Lancs, UK).

Olfactometer Bioassay. A Perspex 4-arm olfactometer, lined on the base with filter paper and lit from above with diffuse uniform lighting was used (Pettersson, 1970). The treated arm inlet tube contained either a single freshly cut wheat panicle in the ear emergence growth stage, or an aliquot of the test solution applied using a micropipette (Drummond "microcaps," Drummond Scientific Co., USA) to a piece of filter paper (4 × 25 mm; solvent allowed to evaporate for one min). In the case of test solutions, either air entrainment samples or authentic compounds, the control arm inlet tubes were treated with the same volume of solvent on the filter paper. Air was drawn through the apparatus at 350 ml/min. Female *S. mosellana*, obtained from the laboratory culture, were transferred individually from the rearing cage into the central chamber of the olfactometer by using a custom made piece of glass tubing (made from a Pasteur pipette heated over a Bunsen burner to remove the narrower end). Time spent and number of entries into each olfactometer arm were recorded with "Olfa" software (F. Nazzi, Udine, Italy) over a 16 min bioassay period during which the olfactometer was rotated through 90° every 2 min to avoid directional effects. Initial bioassays with the air entrainment materials were carried out by using a 1 μl sample volume equating to the level of volatiles obtained from one wheat panicle over a 45 min period. Electrophysiologically active compounds were tested on their own at the same level as in the air entrainment sample and at a dose of 100 ng. They were also tested as synthetic blends formulated to provide the same concentration and ratio as in the air entrainment sample. Mean time spent in and number of entries into treated and control arms were compared using a paired *t* test (Genstat).

Air Entrainment of Wheat Volatiles. Since early anthesis is the most attractive growth stage of wheat for ovipositing female midges (Ding and Lamb, 1999), volatiles from the highly susceptible winter wheat cultivar "Lynx" (J. Oakley, pers. comm.) were entrained at this growth stage. Multiple collections were made with portable equipment (Agelopoulos et al., 1999) from glasshouse grown wheat panicles. Collections were also made from the same wheat cultivar grown in field plots on Rothamsted farm to allow comparison with volatile emission under field conditions.

For each entrainment, three panicles were enclosed in a glass vessel, 100 mm diam and 300 mm in length, open at the bottom and closed with a collection port at the top. The bottom was then closed with two semicircular aluminum plates that fitted around the stem of the plant and were clipped to a flange on the open end of the glass vessel. One of the aluminum plates was drilled to accommodate an inlet port, and air, purified by passage through an activated charcoal filter, was pumped into the vessel through this (400 ml/min). Volatiles were collected on Porapak Q absorbent tubes inserted into the collection ports on the top of the vessels. Further pumps drew air (300 ml/min) through these tubes. Rates were controlled so that more purified air was pumped in than was drawn out, ensuring that unfiltered air was not drawn into the vessel from outside and obviating the need for a tight seal

around the stem, which would have caused damage to the plant. All connections were made with PTFE tubing and ferrules, and as much as possible the equipment, particularly the glassware, was heated at 180°C for at least 2 hr before use. Porapak Q tubes were conditioned at 140°C in a stream of purified nitrogen for at least 4 hr before use (for method, see Blight, 1990). Panicles were entrained for 1 wk to collect sufficient material for subsequent bioassays. Porapak Q filters were eluted with 0.5 ml of redistilled diethyl ether, and the samples collected were stored in vials in a freezer (−20°C).

Electrophysiology. Electroantennogram (EAG) recordings from whole antennae of female *S. mosellana* were made using Ag–AgCl glass electrodes filled with saline solution (composition as in Maddrell, 1969, but without glucose). The abdomen was inserted into the glass capillary (Clark Electromedical Instruments, Reading, UK) of the reference electrode, and both antennae were then directed into the recording electrode so that the distal end was immersed in saline and held in place by surface tension. Signals were passed through a high impedance amplifier (UN-06, Syntech, Hilversum, the Netherlands) and analyzed with a software package (Syntech, the Netherlands). The stimulus delivery system, which employed 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 l/min) flowing continuously over the preparation. Samples (10 μl or 1 μl, respectively) of the air entrainment samples or standard solutions of test compounds were applied to filter paper strips, and the solvent was allowed to evaporate (30 sec) before the paper strip was placed in the cartridge. Fresh cartridges were prepared immediately prior to each stimulation. The control stimulus was solvent (either 10 μl diethyl ether with entrainment samples or 1-μl hexane with standard solutions). Test stimuli were alternated with solvent controls to allow for the decline in EAG response of the preparations with time. For each test stimulus, a mean of the preceding and following control response was calculated, and the test and control responses were then compared using a paired t test (Genstat).

Coupled GC-EAG. The coupled GC-electrophysiology system, in which the effluent from the GC column is simultaneously directed to the antennal preparation and the GC detector, has been described previously (Wadhams, 1990). Separation of the volatiles was achieved on a 50 m × 0.32 mm i.d. HP-1 column fitted in an AI 93 GC equipped with a cold on-column injector and a flame ionization detector (FID). The oven temperature was maintained at 40°C for 2 min and then programmed at 5°/min to 100°C and then at 10°/min to 250°C. The carrier gas was hydrogen (flow rate 42 cm/sec). The outputs from the EAG amplifier and the FID were monitored simultaneously and analyzed with a software package (Syntech, the Netherlands).

GC Analysis. GC analysis was carried out using an Hewlett-Packard 5890 GC equipped with temperature programmable on-column injectors and FIDs and was fitted with two columns of different polarities, a 50 m × 0.32 mm i.d. HP1

(nonpolar) and a 30 m × 0.32 mm i.d. Solgel-Wax (polar). For both columns, the carrier gas was hydrogen (flow rate 44.9 and 55.5 cm/sec, respectively). The oven was maintained at 40°C for 1 min and then programmed at 10°/min to 250°C. Quantification was carried out by comparison with known amounts of authentic standards.

GC-MS. A capillary GC column (50 m × 0.32 mm i.d. HP-1) fitted with a cold on-column injector was directly coupled to a mass spectrometer (VG Autospec, Fisons Instruments, Manchester, UK). Ionization was by electron impact at 70 eV, 250°C. The oven temperature was maintained at 30°C for 5 min and then programmed at 5°/min to 250°C. Tentative *GC-MS* identifications were confirmed by peak enhancement with authentic samples on both the polar and nonpolar GC columns (Pickett, 1990).

RESULTS AND DISCUSSION

In the olfactometer, there was significant attraction ($P < 0.05$, paired t test) of female *S. mosellana* to single freshly cut panicles of the wheat cultivar "Lynx" in ear emergence growth stage, and also to a 1 μl aliquot (equivalent to 45 min emission from one wheat panicle) of the air entrainment sample of "Lynx" volatiles (Figure 1). Six physiologically active components were located in the air entrainment samples, which were identified by GC-MS and coinjection with authentic standards on both polar and nonpolar columns, as acetophenone, (*Z*)-3-hexenyl acetate, 3-carene, 2-tridecanone, 2-ethyl-1-hexanol, and 1-octen-3-ol (Figure 2). Electrophysiological (EAG) activity of the identified compounds was confirmed with the authentic standards (Table 1). Although it is recognized that 3-carene and 1-octen-3-ol are chiral, the low levels of these components and their production in a complex background have so far precluded assignment of their stereochemistry. Therefore, commercially available (+)-3-carene and (±)-1-octen-3-ol were used.

Of the six electrophysiologically active compounds, none was behaviorally active when presented individually at the levels present in the air entrainment sample (see below), although acetophenone, (*Z*)-3-hexenyl acetate, and (+)-3-carene were attractive in the olfactometer at a dose of 100 ng on filter paper (Table 2). A six-component blend comprising all the EAG active compounds, in the same proportion and concentration as in the air entrainment sample, was used for confirmation of behavioral activity (3.4 ng/μl (*Z*)-3-hexenyl acetate, 7.4 ng/μl 3-carene, 1.3 ng/μl acetophenone, 1.7 ng/μl octen-3-ol, 1.8 ng/μl 2-ethyl-1-hexanol, and 607 ng/μl 2-tridecanone). It elicited significant attraction from female midges. However, a three component mixture comprising only 1.3 ng/μl acetophenone, 3.4 ng/μl (*Z*)-3-hexenyl acetate, and 7.4 ng/μl 3-carene was also attractive ($P = 0.007$, Figure 1). Thus, despite the large number of compounds emitted by wheat panicles (Figure 2), host location appears to be mediated by a

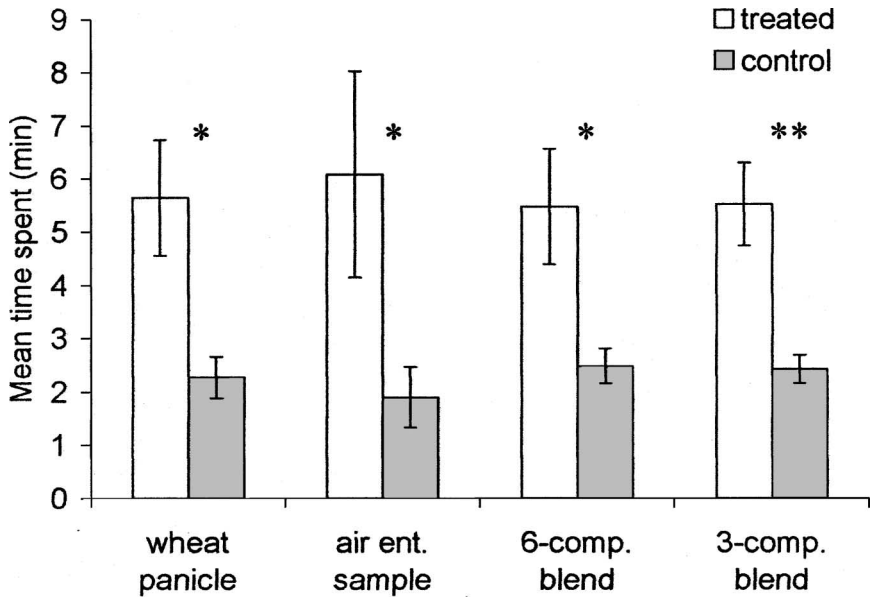

FIG. 1. Response of female *S. mosellana* to wheat (cv. "Lynx") panicle volatiles in the olfactometer: time spent in treated and control arms ($N = 9$ for wheat panicle and air entrained sample; $N = 15$ for 6- and 3-component synthetic blends). *Significantly different ($P < 0.05$). **Significantly different ($P < 0.01$).

limited number of key compounds of which acetophenone, (*Z*)-3-hexenyl acetate, and 3-carene are relatively minor components of the overall volatile profile.

The levels of volatiles produced were, with the exception of 2-tridecanone, very low. Mean emission levels (from seven entrainments) of the electrophysiologically active components were 1.87 ng/panicle/hr of acetophenone, 5.68 ng/panicle/hr of 3-carene, 4.02 ng/panicle/hr of 2-ethyl-1-hexanol, 4.44 ng/panicle/ hr of 1-octen-3-ol, and 1411 ng/panicle/hr of 2-tridecanone. The values are slightly different from the amounts used in the bioassay, which was based on a single air entrainment collection and was at a dose equivalent to 45-min emission from one panicle. In order to ensure that samples collected from glasshouse grown plants reflected a realistic situation, volatiles were also collected from "Lynx" growing under field conditions. GC analysis of these samples showed that there were no significant qualititative or quantitative differences in the production of the electrophysiologically active compounds between glasshouse and field grown plants.

This study presents the first evidence for host location of *S. mosellana* being mediated by plant-derived volatile semiochemical cues. However, it is perhaps

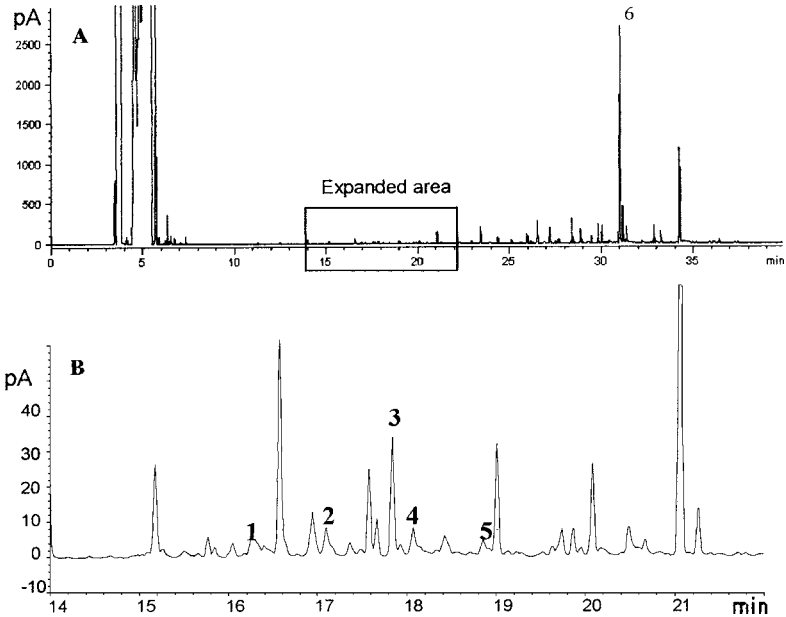

FIG. 2. GC-trace showing cv. "Lynx" volatiles exciting electrophysiological activity from female *S. mosellana* antennae in GC-EAG analyses (1 = 1-octen-3-ol, 2 = (*Z*)-3-hexenyl acetate, 3 = 3-carene, 4 = 2-ethyl-1-hexanol, 5 = acetopnenone, and 6 = 2-tridecanone). Lower trace (B) shows an area expanded from the upper trace (A).

surprising, bearing in mind the cereal specific nature of *S. mosellana*, that the electrophysiologically and behaviorally active compounds mediating this interaction are ubiquitous plant/floral volatiles (Knudsen et al., 1993). 3-Carene occurs less widely than (*Z*)-3-hexenyl acetate and acetophenone, but nevertheless has

TABLE 1. EAG RESPONSES OF FEMALE *S. mosellana* TO COMPOUNDS (10 μG DOSE) IDENTIFIED FROM *T. aestivum* CV. "LYNX" ($N = 8$)

Compound	Corrected EAG response (± SE) [−mV]	*P* value (*t* test)
acetophenone	0.30 (±0.07)	0.002*
(*Z*)-3-hexenyl acetate	0.12 (±0.02)	<0.001*
(+)-3-carene	0.07 (±0.03)	0.023*
2-tridecanone	0.25 (±0.11)	0.029*
2-ethyl-1-hexanol	0.11 (±0.04)	0.013*
1-octen-3-ol (racemic)	0.16 (±0.03)	0.001*

Note. These were correlated by subtracting the response to the hexane solvent control.
*treated significantly different from control ($P < 0.05$).

TABLE 2. RESPONSE OF FEMALE *S. mosellana* TO COMPOUNDS (100 ng ON FILTER PAPER) TESTED INDIVIDUALLY IN OLFACTOMETER BIOASSAY ($N = 8$)

Compound	Treated mean (minutes)	SE	Control mean (minutes)	SE	P value (t test)
(*Z*)-3-hexenyl acetate	5.31	(±0.79)	2.75	(±0.19)	0.009*
2-ethyl-1-hexanol	3.61	(±1.04)	2.96	(±0.46)	0.656
2-tridecanone	2.84	(±0.50)	2.91	(±0.25)	0.897
(+)-3-carene	4.67	(±0.41)	2.58	(±0.54)	0.026*
acetophenone	5.80	(±1.07)	2.46	(±0.25)	0.017*
1-octen-3-ol (racemic)	3.94	(±1.54)	3.34	(±0.47)	0.771

* Treated significantly different from control ($P < 0.05$).

been recorded, for example, in apple flowers (Buckbauer et al., 1993), oilseed rape flowers (Blight et al., 1997), citrus leaf and peal oil (Lota et al., 2001), and in pine needles (e.g., Roussis et al., 1995).

Host-plant selection by phytophagous insects can be seen as a continuum between, at the one extreme, host-plant finding when insects "choose" their host from a distance and, at the other, host-plant recognition when "choice" occurs only after contact (Visser, 1988). In the natural ecosystems in which insects evolved, host plants are often hidden among a complex array of host and nonhost plants. Although some authors have questioned the role of volatile semiochemicals (Finch and Collier, 2000), there is overwhelming and long standing evidence for their role in host location (Kennedy, 1965; Dethier, 1982; Visser, 1986; Bernays and Chapman, 1994; Zhang et al., 1999; Bruce and Cork, 2001; Nojima et al., 2003). Visser (1986) presented two hypotheses relating to the possible role of volatile plant-derived semiochemical cues in host location: (i) that plant odors are highly specific and composed of compounds not found in unrelated plant species, and (ii) that plant odor specificity is achieved by the particular ratio between constituent components that are generally distributed among plant species. The results reported in this work provide some support for the second hypothesis since the active compounds identified are not specific to wheat. However, further studies are required to determine the role of ratios in host recognition by this insect.

S. mosellana is a serious though sporadic pest of wheat. Because of its cryptic nature, forecasting risk to a particular crop is difficult and consequently many farmers adopt a prophylactic approach to control with potentially high economic and environmental costs associated with an inappropriate pesticide application. While the sex-pheromone-based trapping system, which catches male *S. mosellana* (Gries et al., 2000), can provide information relating to midge emergence in a particular area, damage levels in individual fields are likely to be better correlated with number of ovipositing females. Indeed, preliminary field experiments conducted at Rothamsted have shown that numbers of male *S. mosellana* caught in

pheromone traps do not directly relate to infestation levels in adjacent fields. Thus, the identification of kairomones mediating host location by gravid females provides the potential to develop a dual stage monitoring system with the pheromone trap detecting midge emergence and triggering deployment of a plant semiochemical based system to determine the need to treat specific crops passing through the susceptible growth stages.

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VOLATILES PRODUCTION AND ATTRACTIVENESS TO THE MEXICAN FRUIT FLY OF *Enterobacter agglomerans* ISOLATED FROM APPLE MAGGOT AND MEXICAN FRUIT FLIES

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Abstract—We investigated two strains of uricase (+) *Enterobacter agglomerans*, one isolated from the apple maggot fly (AMF) and one from the Mexican fruit fly (MFF), for 1) attractiveness to MFF, and 2) production of attractive chemicals. Regarding chemicals demonstrated attractive to the MFF, the MFF bacterial strain produced more 2,5-dimethylpyrazine, 2-phenylethanol, and indole than the AMF strain, whereas the AMF, but not the MFF strain, produced 3-hydroxybutanone. Cell types that predominated in plated subcultures varied from batch to batch resulting in variation in volatiles production, especially by the AMF strain where indole was sometimes a major component of the odor and at other times not detectable. Despite the greater production of attractive chemicals by the MFF strain, the AMF strain was consistently more attractive and the MFF strain was not different from uninoculated control plates. Statistical analyses indicated negative correlations of attractiveness with production of indole, 2,5-dimethylpyrazine, and 2-phenylethanol, and positive correlation with 3-hydroxybutanone. Results support previous findings with the Mexican fruit fly that showed combinations of attractive chemicals sometimes are not attractive.

Key Words—Attractants, Diptera, Tephritidae, *Anastrepha ludens*, ammonia, 3-methylbutanol, 3-hydroxybutanone, indole, solid phase microextraction (SPME).

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INTRODUCTION

During the past 20 years, numerous bacteria associated with fruit flies of the family Tephritidae have been identified (Fitt and O'Brien, 1985; Drew and Lloyd, 1989; Howard, 1989; Jang and Nishijima, 1990; MacCollom et al., 1992; Martinez et al., 1994; Kuzina et al., 2001; Marchini et al., 2002). In many cases, bacteria have been demonstrated to be attractive to the flies from which they were isolated (Jang and Nishijima, 1990; Robacker et al., 1991; MacCollom et al., 1992; Martinez et al., 1994). Various roles of these bacteria in the natural history of the flies have been proposed, including one as obligate symbiotes that flies transmit through various life stages (Petri, 1910; Allen and Riker, 1932; Hagen, 1966), facultative symbiotes that may convert unusable nitrogenous compounds into usable nitrogen (Lauzon et al., 2000), accidental symbiotes that are picked up by the flies while feeding (Huston, 1972; Howard, 1989), a source of protein (Drew et al., 1983; Drew and Lloyd, 1989), agents that detoxify unwanted components in food (Boush and Matsumura, 1967), or odor-producing indicators of proteinaceous foodstuffs to which flies are attracted (Robacker and Moreno, 1995). Bacteria could serve any one or several of these roles given different fruit fly species, bacteria species, and environmental and physiological circumstances.

A bacterium that has been closely tied to the natural history of the apple maggot fly (AMF), *Rhagoletis pomonella*, is *Enterobacter agglomerans*. Several strains of this bacterium were identified from AMF, their environment, and bird feces fed on by AMF (Lauzon, 1988, 1991; MacCollom et al., 1992, 1994; Lauzon et al., 1998). One strain proved highly attractive to AMF in field tests where it performed as well as apple volatiles and increased overall attraction when it was combined with apple volatiles on Ladd traps (MacCollom et al., 1992). The same strain outperformed both ammonium acetate and another strain of *E. agglomerans* obtained from the ATCC culture collection (MacCollom et al., 1994). Another strain of *E. agglomerans* isolated from apple leaf samples taken from the habitat of AMF proved more attractive than a strain isolated from chicken feces (Lauzon et al., 1998). Finally, uricase (+) strains of both *E. agglomerans* and *E. cloacae* isolated from AMF alimentary canals were more attractive to AMF than uricase (−) strains of each species (Lauzon et al., 2000). These studies confirmed the hypothesis of Prokopy et al. (1993) that bird feces containing microorganisms could generate volatiles more attractive to AMF than feces that did not contain them, although the specific ability to metabolize uric acid to ammonia was not discussed.

Initial chemical analyses of one AMF strain attractive to the AMF (later determined uricase (+)) were carried out by Epsky et al. (1998). They showed that this uricase (+) strain was attractive to the Caribbean fruit fly (*Anastrepha suspensa*), quantified the release of ammonia and 3-methylbutanol from the bacterial cultures, and demonstrated that combinations of these two chemicals were more

attractive to the flies than either alone. Robacker and Lauzon (2002) tested attractiveness to the Mexican fruit fly (MFF) of this uricase (+) strain and a uricase (−) strain isolated from AMF. They showed that the uricase (+) strain was the more attractive and that the two strains produced qualitatively and quantitatively different volatiles as a result of metabolizing a culturing medium that contained uric acid as its principal nitrogen source.

Having demonstrated differences in attractiveness to MFF and volatiles produced by uricase (+) and uricase (−) strains of *E. agglomerans* (Robacker and Lauzon, 2002), C. R. Lauzon hypothesized that a bacterial strain isolated from a particular species of fruit fly might be more attractive to that species than a strain isolated from another fly species. To investigate this hypothesis, a uricase (+) strain of *E. agglomerans* was isolated from the MFF for comparison with the same uricase (+) AMF strain used in the work of Robacker and Lauzon (2002). Our objectives were to 1) test attractiveness of each strain to MFF; 2) identify and compare volatiles produced by the two strains; and 3) relate attractiveness of each strain with production of volatiles.

METHODS AND MATERIALS

Insects and Test Conditions. Mexican fruit flies were from a culture that originated from yellow chapote fruit (*Casimiroa greggii*, S. Wats. F. Chiang), a native citrus host of the fly, collected in Nuevo Leon, Mexico, in 1997. Mixed-sex groups of 20–25 flies (overall sex ratio 1:1, but individual cups varied) were kept in 473-ml cardboard cartons with screen tops until used in tests. Cups were provisioned with separate sugar and water sources, but no protein. Laboratory conditions for holding and testing flies were 22 ± 2°C, 50 ± 20% relative humidity, and photophase from 0630 to 1930 hr. All tests were conducted between 0900 and 1600 hr with 2–27 day-old flies. Previous work demonstrated that attraction of MFF to odors of aqueous bacterial cultures was maximum for sugar-fed, protein-deprived flies compared with other feeding regimens, and uniform between 0830 and 1630 hr under these temperature and relative humidity conditions for flies between 2–15 days post eclosion (Robacker and Garcia, 1993). However, responsiveness decreased by about 50% as flies aged from 15 to 30 days.

Bacterial Cultures and Biochemical Tests. A strain of *Enterobacter agglomerans* isolated from the midgut of wild MFF, and a strain of *E. agglomerans* isolated previously from AMF (Lauzon et al., 2000) were grown individually in tryptic soy broth (Difco Laboratories, Detroit, MI). Growth rates were monitored and compared over a 24-hr period. Growth rates were similar between the two strains, however, *E. agglomerans* cells from MFF displayed flocculent growth not observed for *E. agglomerans* from AMF. Bacterial cells were prepared for plating as described in Robacker and Lauzon (2002). Briefly, each strain was grown in nutrient medium for 18 hr at 28°C, centrifuged, and washed free of medium. Cells

were adjusted to similar optical densities (≅0.550, $\lambda = 550$ nm), plated on a uric acid medium, and sent via overnight delivery service to the USDA-ARS laboratory in Weslaco, TX for volatiles analysis and insect bioassays. After all bioassay and identification work was finished, we conducted biochemical tests using API 20E biochemical identification strips (BioMerieux, Marcy-l'Etoile, France) on both bacterial strains to determine possible reasons for changes in volatiles production that had been observed.

Wind-Tunnel Bioassay. Bioassays were conducted in a Plexiglas wind tunnel with the dimensions of $0.3 \times 0.3 \times 1.2$ m. Each end of the tunnel was screened to allow airflow. The downwind end contained a baffle system to create a uniform airflow through the chamber. Air was pulled through the chamber at 0.4 m/sec by an exhaust fan connected to the downwind end. Air exiting the chamber was directed into an exhaust hose and removed to the outdoors. In addition to the direct exhaust from the wind tunnel, this room contained inlet and outlet vents to bring in new air from the outdoors and remove air to the outdoors. Complete air replacement in the room occurred 8×/hr.

The top of the chamber had two circular openings (12.8 cm diam) with Plexiglas covers, located at each end of the chamber, to allow easy access to the interior. A 75 W "soft white" light bulb (General Electric Co., Cleveland, OH) in a reflecting lamp was positioned 17 cm above the downwind end. The purpose of this light was to minimize random flying into the upwind end by using the flies' positive phototactic reaction. Overhead lighting was provided by fluorescent "cool white" lights (F40CW, General Electric).

Attractiveness of plates containing either active cultures or uninoculated medium was assessed. Plates were incubated after arrival in Weslaco for 1–10 days at 30–32°C prior to testing. Most assays were conducted using incubations of 5–10 days because observations indicated that uric acid utilization was maximum during this period (judged by disappearance of uric acid crystals in medium). To conduct a trial, a plate bottom containing the active culture or uninoculated medium was placed on the floor of the chamber below the opening at the upwind end. One cup of flies was placed under the downwind opening. Flies were given 5 min to leave the cup, fly or walk upwind, and contact the plate. Upwind movement was scored if flies passed a point 2/3 of the distance from the release cup to the plate.

Sixteen batches of bacterial plates were tested. Each batch was divided into two groups that were bioassayed on different days. On 20 test days, test flies comprised four age/feeding status groups: young (2–5 days post eclosion), sugar-fed; young, sugar-deprived (for 1 day); old (12–27 days), sugar-fed; and old, sugar-deprived. Each plate type (AMF strain, MFF strain, uninoculated control) was tested equally with the four age/feeding status groups. Five–six-day-old, sugar-fed flies were tested on 8 day, 5–6 day-old, sugar-deprived (1 day deprivation) flies were tested on 2 day, and 19–27 day-old, sugar-fed and sugar-deprived flies were tested on 2 day.

Quantification of Volatiles from Cultures. Volatiles in the headspace above active bacterial cultures and above uninoculated medium were quantified by gas chromatography (GC). Plates used in these analyses were selected from every batch of inoculations that were used for bioassays. Incubation times ranged from 1 to 10 days at 30–32°C. Volatiles were sampled using solid phase microextraction (SPME) with a polydimethylsiloxane (PDMS) coated fiber (100 μm coating) (Supelco, Inc., Bellefonte, PA). The fiber was inserted into the headspace through a small hole drilled into the side of each plate just prior to volatiles collection. The hole was only slightly larger than the fiber sheath to minimize escape of volatiles. Sampling time was 1 hr at 30–32°C. On-column injection of volatiles was by thermal desorption from the SPME fiber at 220°C in a 10 cm retention gap (0.53 mm ID deactivated fused-silica) connected to the analytical column by a GlasSeal™ connector (Supelco). The analytical column was a DB-1 (60 m, 0.32 mm ID, 5 μm film) (J & W Scientific, Folsom, CA). Column oven temperature was 50°C for 5 min, then programmed at 5°C/min to 200°C. Carrier gas was helium at a linear velocity of 40 cm/sec. Analyses were conducted with a Shimadzu GC-17A (Shimadzu Scientific Instruments, Inc., Columbia, MD) equipped with both flame ionization (FID) and flame thermionic (Model FTD-17) detectors. Detection was by FID for most plates, to analyze ammonia and organic chemicals that did not contain nitrogen. Detection was by FTD for others, to analyze organic chemicals containing nitrogen. GC peak areas were measured by using Millennium 2010 Chromatography Manager software (Waters Corporation, Milford, MA).

Peak areas were converted to headspace concentrations in the Petri plates using K values of the chemical analytes on PDMS fibers (Bartelt, 1997). K is defined as the amount of chemical on the PDMS fiber at the end of sampling divided by the concentration in the container at the end of sampling when the system is at equilibrium. K values at 25°C for several of the volatile chemicals identified in this study were published by Bartelt (1997), along with an equation for converting them into K values at 30°C, the incubation temperature used in this study. It was necessary to determine K values for ammonia, indole, 2-undecanone, and 2-tridecanone, also. For 2-undecanone and 2-tridecanone, K values were calculated using their Kovats indices and correction factors provided in Bartelt (1997).

For determinations of K for ammonia and indole, SPME analyses were conducted in 0.5 and 1 liter round-bottom flasks containing various amounts of the chemicals introduced in methanol solutions at 30°C. Flasks were capped with parafilm, and the air was stirred with a magnetic stir bar for 0.5 hr before the PDMS fiber was inserted through the parafilm and volatiles were collected for 1 hr. FID areas were measured using GC methods described above. Calibration curves for the two chemicals were made by syringe injections of methanolic solutions into a splitless injector. Other GC conditions were the same as for the SPME analyses. The on-column injector was not used for the solvent injections because

baseline noise prevented reliable measurement of small peaks like those produced by the ammonia standards. A comparison of FID areas obtained by splitless injection versus on-column injections was made by using higher concentrations of ammonia, indole, and several other chemicals. On-column injections yielded peak areas approximately 4× greater than splitless injections. This factor was used to correct FID values obtained by splitless injection before using them to produce calibration curves. Calibration curves were used to determine amounts of ammonia and indole on the PDMS fiber after sampling in the round bottom flasks, and these values were used to calculate K values of ammonia and indole. K values determined in this way were similar to those calculated using the equation in Bartelt (1997) where it was assumed that ammonia contains an amino group and that indole has a pyridine-like functionality.

For all chemicals, amounts on the fiber at the end of sampling Petri plates containing bacterial cultures were determined using calibration curves as described above in which syringe injections of standards were made into a splitless injector, but response factors were adjusted by 4× to quantify SPME injections of plate samplings in the on-column injector. Headspace concentrations at the end of sampling were calculated as the amount on the fiber divided by K (units of K are ml). The amount of each chemical in the headspace before sampling was calculated as the product of the concentration after sampling multiplied by 30 ml (approx. headspace volume of Petri plates), then adding the amount on the fiber at the end of sampling. The concentrations in the Petri plates before SPME sampling were calculated as the quotient of the amount in the headspace before sampling divided by 30 ml.

Identification of Volatiles Produced by Bacteria. Volatiles produced by bacteria were identified by gas chromatography/mass spectrometry (GC–MS). The method described above in which volatiles were sampled from plates using SPME PDMS was used for GC–MS analysis of three plates each of the AMF and MFF strains. The fiber was exposed inside the plates for 1 or 24 hr. Twenty-four hour samplings were done to collect sufficient amounts of the minor components for positive identification. For injection, chemicals were thermally desorbed from the fiber for 1 min in a split/splitless injector in the splitless mode at 250°C. The injector was purged after 1 min. The analytical column for GC–MS was the same DB-1 column described above. Linear velocity of helium carrier gas was 40 cm/sec. Column oven temperature was programmed from 50 to 230°C at 5°C/min, and holding at the final temperature for 19 min. GC–MS data were acquired using Hewlett-Packard 6890 GC (Hewlett-Packard Company, San Fernando, CA) with an HP 5973 Network Mass Selective Detector (EI) (electron energy = 70 eV) over a mass range of 40–550 amu. The system was controlled by an HPMS Chemstation. GC–MS identifications were based on computer matching of unknown spectra with those in the NIST 98 Library of Mass Spectra and Subsets (Hewlett-Packard).

Identification of Indole. GC-FTD indicated a large unknown peak containing nitrogen. GC–MS spectra yielded nearly equivalent matches with indole and two other compounds. Retention times of indole and the unknown in volatiles collected by SPME from a MFF strain plate were compared after injection onto the DB-1 column and onto a SupelcoWAX-10 capillary column (60 m, 0.32 mm ID, 0.25 μm film) (Supelco) using the conditions described above. The other two candidate chemicals were not commercially available for testing.

Acetyl chloride derivatization was also used to differentiate among the three candidate chemicals based on its reactivity with secondary amines such as indole and nonreactivity with tertiary amines such as the other two compounds. For this procedure, cells and some media were scraped from three MFF plates and extracted in 2 ml of dichloromethane. Ten drops of acetyl chloride (Sigma Chemical Co., St. Louis, MO) were added to a mixture containing 200 μl of the cell extract, 200 μl of pyridine, and 400 μl of benzene. The solution was refluxed for 1 hr at 65–70°C, then 1 μl of the reaction mixture was analyzed by GC on the DB-1 column.

Volatiles Collection on Activated Charcoal. Because of uncertainty that all important chemicals were detected by SPME, volatile collections were also conducted using activated charcoal. Previous findings indicate that some chemicals that do not bind well to SPME PDMS fiber coatings do bind well to activated charcoal (Lee et al., 1995; DeMilo et al., 1996; Robacker and Bartelt, 1997). For these analyses, an additional batch (batch 17) of AMF and MFF strain plates was prepared, and volatiles from these plates were pulled through ORBO^{TM} 100 activated charcoal tubes (Supelco, Bellefonte, PA) for 24 hr at 30–32°C with a flow rate of 400 ml/min. Tubes were extracted with 2 ml of acetone, and the extracts were concentrated to 100 μl for GC analysis using the same column and conditions that were used for SPME analyses. Five replications were conducted for each bacterial strain.

Chemical Attractiveness Bioassay. Cage-top bioassays as described in Robacker and Flath (1995) were used to test attractiveness of indole in bacterial volatiles. Indole (99%) was obtained from Fluka (Milwaukee, WI). Four quantities were tested in methanol: 10 ng, 100 ng, 1 μg, and 10 μg. The bioassay was conducted by placing two filter paper triangles (3 cm/side) containing 10 μl of indole solution and two papers containing 10 μl of methanol near the corners on top of an aluminum-screened cage (30 cm/side) containing 180–200 adult flies. Papers containing indole were positioned diagonally from each other on the cage top, as were the papers containing methanol. Papers were raised 5 mm above the cage top using plastic rings to ensure that olfaction was solely responsible for responses. The number of flies beneath each paper was counted once each minute for 10 min following application of test chemicals.

Bioassays were conducted using flies of two feeding regimes: sugar-fed, protein-starved (from eclosion); and sugar-starved (for 1 day), protein-starved.

The four concentrations of indole were bioassayed in random order. Each concentration was assayed 24–36 times with both sugar-fed and sugar-starved flies.

Statistical Analyses. Effect of bacterial strain on headspace concentrations of volatiles was analyzed using SuperANOVA (Abacus Concepts, 1989). Analyses of variance used randomized complete block designs. Separate analyses were conducted for each chemical. Attractiveness of the two strains of bacteria in wind-tunnel bioassays was also tested by analysis of variance using SuperANOVA. For analysis, proportions of flies that moved upwind or contacted the plates in each bioassay were transformed by arcsin of the square root, because means were highly correlated with variances (Snedecor and Cochran, 1967). Proportions of 0 were replaced with $1/(4N)$. Separate analyses were conducted for males and females of different age/feeding status groupings. Means separations were done by Fisher's protected least significant difference (LSD) method in all ANOVAs.

GC area counts of several headspace chemicals from AMF strain plates that produced indole were compared with counts from AMF strain plates that did not produce indole by using *t* tests. Attractiveness of AMF strain plates from batches that produced indole was also compared with that of AMF strain plates that did not by using *t* tests.

Linear regression (SuperANOVA) was used to relate attractiveness of plates with headspace concentrations (FID area counts) of chemicals in plates. For these analyses, attractiveness indices were calculated for both males and females that responded to each bacterial strain on each test day. For example, the attractiveness index for males responding to AMF strain plates on a particular test day was calculated as follows: (the total males that flew upwind toward AMF strain plates + 3× the total males that contacted the plates)/the total males tested that day with AMF strain plates.

Paired *t* tests were used to analyze results of cage-top bioassays that evaluated attractiveness of indole. For paired *t* tests, the total of 10 counts of flies at filter papers containing test chemicals were compared with the total at papers containing solvent for each bioassay.

RESULTS AND DISCUSSION

Chemical Identifications and Quantifications. GC-FID and GC-FTD analyses of SPME volatiles collections revealed about 100 chemicals in the headspace of the bacterial plates. All chemicals considered important in previous work with the AMF uricase (+) strain were found. No peaks with areas greater than 1 mV were found by using activated charcoal that were not found by using SPME. Thus, no peaks found by using activated charcoal were quantified.

Chemicals considered important in previous work (Robacker and Lauzon, 2002) were requantified. Additional peaks were considered if the following three conditions were met: 1) peak areas were at least 1 mV; 2) peaks were found

in at least three different volatiles samplings; and 3) peaks were found in significantly greater amounts in at least one of the two strains than in the uninoculated controls. Chemicals identified by GC–MS that met these criteria were 3-hydroxybutanone, 3-methylbutanol, 2,5-dimethylpyrazine, 2-phenylethanol, 2-undecanone, and 2-tridecanone. Ammonia, identified by GC-FID only, also was quantified. Four additional chemicals met the criteria, but could not be identified by GC–MS either because of nonunique spectra or low peak areas. No attempt was made to identify three of the unknowns that had peak areas just above the 1 mv threshold.

The other unknown yielded large peaks and contained nitrogen as indicated by FTD/FID peak area ratios (8:1). GC–MS indicated a molecular weight of 117.06 and molecular formula of C_8H_7N, but could not distinguish among indole, 5H-1-pyridine, and indoline. Retention times of the unknown on DB-1 and SupelcoWAX-10 columns matched those of indole. Extracts of MFF strain culture treated with acetyl chloride showed a 100% reduction in GC peak area of the unknown compound. Reflux of controls without acetyl chloride showed no reduction in the unknown compound. This demonstrated that the unknown was a primary or secondary amine. As indole is a secondary amine and 5H-1-pyridine and indoline are tertiary amines that do not react with acetyl chloride, this indicated the unknown was indole.

GC-FID quantifications of headspace chemicals are shown in Table 1. Data are headspace concentrations calculated using the method of Bartelt (1997). These were calculated because they represent amounts of the chemicals in the headspace better than do peak areas. However, these concentrations should be viewed only as approximations due to many assumptions that were made, and because concentrations vary with cell count, incubation time and temperature, culturing medium variability, and other factors.

Both bacteria strains produced more ammonia, 3-methylbutanol, 2,5-dimethylpyrazine, 2-phenylethanol, and indole than uninoculated controls. In fact, no 3-methylbutanol, 2-phenylethanol, or indole was found in uninoculated controls. The MFF strain produced more 3-methylbutanol, 2,5-dimethylpyrazine, 2-phenylethanol, and indole than the AMF strain. However, the AMF strain produced 3-hydroxybutanone, a chemical that was not found in headspace of either the MFF strain or uninoculated controls. All of these chemicals except indole had been identified previously from bacterial odors attractive to fruit flies (Gow, 1954; Drew and Fay, 1988; Lee et al., 1995; Robacker and Flath, 1995; DeMilo et al., 1996; Robacker and Bartelt, 1997; Epsky et al., 1998; Robacker et al., 1998; Robacker and Lauzon, 2002). Indole has been reported from *E. agglomerans* (11–25% of strains) and other Enterobacteriaceae (Brenner, 1984; Yu et al., 2000). In addition, 2-undecanone and 2-tridecanone are also known from volatiles produced by *E. coli* (Yu et al., 2000), a bacterium closely related to *E. agglomerans* (Lauzon et al., 1998).

TABLE 1. CONCENTRATIONS OF CHEMICALS IN HEADSPACE OF PETRI PLATE CULTURES OF TWO STRAINS OF *E. agglomerans*

Bacteria strain	*N*	NH3	3HyB	3MeB	25DMP	2PhEt	Indole
Uninoculated medium[a]	15	2.7a (±0.9)	0.0a (±0.0)	0.0a (±0.0)	0.07a (±0.02)	0.0a (±0.0)	0.0a (±0.0)
AMF strain[a]	15	330b (±160)	6.8b (±1.1)	47b (±10)	0.22b (±0.04)	0.33b (±0.07)	4.4b (±1.8)
With indole[b]	6	220 (±71)	5.8 (±2.1)	77* (±19)	0.31 (±0.07)	0.45 (±0.08)	11* (±3.2)
Without indole[b]	9	400 (±250)	7.4 (±1.2)	27* (±3.6)	0.16 (±0.04)	0.24 (±0.09)	0.0* (±0.0)
MFF strain[a]	15	220b (±59)	0.0a (±0.0)	94c (±14)	0.36c (±0.05)	0.55c (±0.07)	8.7c (±1.2)

[a] Mean concentrations (ng/ml) (±SE) for a chemical followed by the same letter were not significantly different from each other by Fisher's protected LSD ($P < 0.05$) based on ANOVAs of FID peak areas measured from each GC determination. Standard errors denote sampling error from determination of FID peak areas; they do not estimate error from determination of concentrations from peak areas.

[b] Significant differences in headspace concentrations between replications of AMF strain plates that produced indole vs. replications that did not produce indole were determined using *t*-tests and are indicated by an asterisk (*).

Of the 16 batches of plates prepared over approximately 1-year duration of this work, nine of the AMF strain batches produced indole and six did not (Table 1). Batches of plates that produced indole were not randomly distributed over time but clumped. Batches 1–2 did not produce indole, batches 3–6 did, batches 8–13 did not (batch 7 was not tested by GC), batches 14–15 did, and batch 16 did not. All of the MFF strain plates produced indole. For batches in which AMF strain plates produced indole, headspace concentrations of indole in AMF strain plates were positively correlated with indole concentrations in MFF strain plates ($r = 0.96$, $df = 8$, $P < 0.001$).

Data in Table 1 were partitioned to compare headspace concentrations of various chemicals for AMF strain plates that produced indole versus those that did not. AMF plates that produced indole also produced other chemicals, except 3-hydroxybutanone, in amounts similar to those produced by the MFF plates. This was most notable for 3-methylbutanol that was found in significantly higher concentrations in AMF plates that produced indole compared with those that did not. 3-Hydroxybutanone was produced nearly equally in AMF plates that produced versus did not produce indole.

2-Undecanone and 2-tridecanone were not included in Table 1 because their headspace concentrations were very low, and no simple ketones are known to be attractive to MFF. The headspace concentrations of 2-undecanone were 33 ± 11 (SE) and 79 ± 21 pg/ml in AMF strain and MFF strain plates, respectively. The headspace concentrations of 2-tridecanone were 18 ± 3.5 and 21 ± 6.1 pg/ml in AMF strain and MFF strain plates, respectively.

Generally, concentrations of chemicals were comparable to those reported in previous studies of volatiles produced by bacteria attractive to the MFF (Robacker and Flath, 1995; Robacker and Bartelt, 1997; Robacker et al., 2000). However, the concentration of ammonia was lower than that found in most previous work and notably was lower than that reported in our previous work with the same AMF uricase (+) strain of *E. agglomerans* (Robacker and Lauzon, 2002).

Attractiveness of AMF and MFF Strains. On the 20 test days in which four age/feeding status groups of flies were bioassayed, older females flew upwind toward ($F = 10.4$; $df = 1,195$; $P < 0.01$) and landed on ($F = 9.7$; $df = 1,195$; $P < 0.01$) plates (three plate types combined) more often than younger females. Age did not affect male responses. Hunger status had no effects on either males or females. Interactions of age and hunger status with bacterial strain (AMF vs. MFF strains or replications of the AMF strain that produced indole vs. those that did not) were not significant indicating that fly age and hunger status did not affect responses to the different strains. Therefore, all bioassay results were combined for further analysis without regard to fly age or sugar-feeding regime.

Summed over all bioassays, more males and females flew upwind toward and landed on AMF strain plates than on either uninoculated or MFF strain plates ($F = 14.8$, 12.7, 22.9, 17.5 for males upwind, females upwind, males landing,

TABLE 2. ATTRACTION OF MEXICAN FRUIT FLIES TO PETRI PLATES CONTAINING TWO STRAINS *E. agglomerans* OF IN A WIND TUNNEL

	Bacteria strain	*N*	Upwind movement	Contact with plate
Males	Uninoculated medium[a]	101	11.4 ± 1.0a	3.2 ± 0.5a
	AMF strain[a]	109	18.7 ± 1.3b	7.2 ± 0.8b
	With indole[b]	35	14.9 ± 1.9*	3.9 ± 1.0*
	Without indole[b]	67	21.1 ± 1.8*	9.1 ± 1.1*
	MFF strain[a]	109	11.5 ± 1.0a	1.8 ± 0.4a
Females	Uninoculated medium[a]	101	11.0 ± 1.0a	2.6 ± 0.6a
	AMF strain[a]	109	17.7 ± 1.3b	6.9 ± 0.8b
	With indole[b]	35	18.9 ± 2.4	6.8 ± 1.6
	Without indole[b]	67	17.3 ± 1.6	7.0 ± 1.0
	MFF strain[a]	109	11.0 ± 1.0a	2.3 ± 0.5a

[a] Values are mean percentages (±SE) responding out of the total flies in the trial. N = trials per treatment. Means in the same sex and same column followed by different letters are significantly different at the 5% level by Fisher's protected LSD.

[b] Significant differences in responses between replications of AMF strain plates that produced indole vs. replications that did not produce indole were determined using t-tests and are indicated by an asterisk (*).

females landing, respectively; $df = 2,301$; $P < 0.001$) (Table 2). Responses to the MFF strain were not significantly different from responses to uninoculated controls using the complete model. However, fewer males landed on MFF strain plates than on uninoculated plates using a model that did not include AMF strain plates ($t = 2.3$, $df = 193$; $P < 0.05$). Among AMF strain plates, those that produced indole were less attractive to males than those that did not (upwind movements: $t = 2.2$, $df = 100$; $P < 0.05$; landings: $t = 3.1$, $df = 100$; $P < 0.01$). Effects of indole on responses of females were not evident.

Bioassays of Indole. Attractiveness of indole in cage-top bioassays was significant by paired t tests ($P < 0.05$) for starved flies at 10 ng, 100 ng, and 10 μg test quantities. Differences between counts at filter papers containing indole and control papers with solvent (methanol) were positive for all test quantities, and no dose–response relationship was evident so data were combined over test quantities resulting in mean counts (per bioassay) of flies at papers containing indole of 35.2 ± 1.0 (SE) ($N = 120$ bioassays) and mean counts at solvent papers of 29.3 ± 1.0. The difference was significant by a paired t test ($t = 5.2$, $df = 118$, $P < 0.01$), indicating that indole was attractive to sugar-starved flies.

Indole was not significantly attractive to sugar-fed flies at any individual test quantity although the difference between counts at papers containing indole and solvent papers was positive at the lowest three test quantities (10 ng, 100 ng, 1 μg). Assuming that the highest test quantity (10 μg) was too high to be attractive, data were combined over the three lowest test quantities for analysis. For this combination, mean counts (per bioassay) of flies at papers containing indole

were 16.6 ± 0.9 (SE) ($N = 84$ bioassays) and mean counts at solvent papers were 14.0 ± 1.0. The difference was significant by a paired t test ($t = 2.4$, $df = 82$, $P < 0.05$), indicating that indole was attractive to sugar-fed flies.

These results show that indole is somewhat attractive to MFF. For sugar-starved flies, counts at indole papers were only 20% greater than counts at solvent papers. For sugar-fed flies, counts at indole papers (three lowest test quantities) were only 19% greater than counts at solvent papers. These results suggest that indole probably would not play a major role in attractiveness of the bacterial plates.

Lack of Attractiveness of MFF Strain Plates. MFF strain plates produced more ammonia, 3-methylbutanol, 2,5-dimethylpyrazine, 2-phenylethanol, and indole, than uninoculated plates. However, MFF strain plates were not more attractive than uninoculated plates to females, and were less attractive to males (Table 2, discussion above). As we found no other chemicals produced by the two plate types that differed significantly in emissions, the most prudent conclusion is that one or more of the chemicals produced by the MFF strain plates inhibited attraction.

To determine if chemicals produced by MFF strain plates inhibited attractiveness, we analyzed linear regression of attractiveness indices (% of flies to move upwind + 3×% of flies to contact the plates, per test day) versus headspace concentrations of ammonia, 3-methylbutanol, 2,5-dimethylpyrazine, 2-phenylethanol, and indole in MFF strain plates. No models with one or more of the chemicals were significant for either male or female indices. We considered that lack of significant correlations did not invalidate the inhibition hypothesis because MFF strain plates were not actually attractive, such that whatever apparent differences occurred in attractiveness indices were probably due to random variation.

We reasoned that if inhibition by one or more chemicals could be shown in the AMF plates, then the same effect could account for the low attractiveness of the MFF plates. For AMF strain plates, the linear regression of attractiveness indices of males versus indole was significant ($r = -0.52$, $df = 23$, $P < 0.01$). Recall also that fewer males moved upwind toward and contacted AMF strain plates that produced indole compared with plates that did not produce indole (Table 2). No other models with one or more of the other four chemicals were significant for male attractiveness indices. No models containing one or more of these five chemicals were significant for female attractiveness indices. The best fit was a negative correlation between attractiveness index and headspace concentration of indole ($r = -0.33$, $df = 23$, $P = 0.1$). These results suggest that indole may have inhibited attraction to the AMF strain plates.

Assuming that the five chemicals under consideration were the only ones affecting attractiveness of plates, we combined data for AMF and MFF strain plates. Negative correlations of indole concentration with attractiveness indices were significant for both males and females (males: $r = -0.52$, $df = 48$, $P < 0.001$; females: $r = -0.34$, $df = 48$, $P < 0.05$). Negative correlations were also significant for 2,5-dimethylpyrazine (males: $r = -0.36$, $df = 28$, $P = 0.05$; females:

$r = -0.49$, $df = 28$, $P < 0.01$) and 2-phenylethanol (males: $r = -0.44$, $df = 28$, $P < 0.05$; females: $r = -0.45$, $df = 28$, $P < 0.05$). Multiple regression models did not account for significantly greater variation in attractiveness index for either males or females. Headspace concentrations of indole were positively correlated (but not significant at the 5% level) with concentrations of 3-methylbutanol, 2,5-dimethylpyrazine, and 2-phenylethanol. Further, headspace concentrations of 3-methylbutanol, 2-phenylethanol, and 2,5-dimethylpyrazine were positively correlated with each other (smallest $r = 0.64$, $df = 13$, $P = 0.05$). These results suggest that several of these chemicals may have acted together to inhibit attractiveness.

Bioassays of indole indicated that it was slightly attractive to both sugar-fed and sugar-starved flies. Also, ammonia (Robacker et al., 2000), 2,5-dimethylpyrazine (Robacker and Warfield, 1993; Robacker and Flath, 1995), and 2-phenylethanol (Robacker and Lauzon, 2002) are all known to be attractive to MFF. These results apparently contradict the analyses showing negative correlations of one or more of these chemicals with attractiveness.

However, there is precedence for attractive chemicals inhibiting other attractive chemicals in the MFF. As examples, pyrrolidine (Robacker and Warfield, 1993), dimethylamine, ethylamine, and 2,5-dimethylpyrazine (Robacker et al., 1997), acetic acid (Robacker et al., 1996), and a mixture of 1,8-cineole, hexanol, ethyl hexanoate, and ethyl octanoate (Robacker and Heath, 1997), each inhibited attraction of MFF to various attractive mixtures containing ammonia and other amino compounds, even though each of these inhibitory chemicals was attractive when tested alone. Also, chemicals that are attractive at low concentrations can become repellent at higher ones. Examples of this effect are putrescine (Robacker and Warfield, 1993) and ammonia (Robacker et al., 1997) that each enhanced attractiveness in mixtures containing them and methylamine. Each, however, also depressed attractiveness if their concentrations were raised to levels that actually were most attractive when each chemical was tested by itself. There was a suggestion that indole may have enhanced attractiveness of AMF strain plates to females, as its headspace concentration increased from 0 (attractiveness index = 0.4) to about 5 ng/ml (index = 0.7), then depressed attractiveness as its concentration increased to about 20 ng/ml (index = 0.1), but the effects were not significant.

Attractiveness of AMF Strain Plates. AMF strain plates were significantly more attractive than MFF strain plates even though MFF strain plates had higher concentrations of 2,5-dimethylpyrazine, 2-phenylethanol, and indole. AMF strain plates, but not MFF strain plates, produced 3-hydroxybutanone, another chemical attractive to this fly (Robacker and Lauzon, 2002). Concentrations of ammonia in the headspace of the two strains were not significantly different.

Considering that headspace of each plate type contained the same chemicals except 3-hydroxybutanone, two explanations for these results can be hypothesized: 1) higher concentrations of some of the attractive chemicals in the MFF plates inhibited attraction relative to the AMF strain plates; and/or 2) 3-hydroxybutanone

increased attractiveness of AMF strain plates. The first explanation was verified as a possibility in regression analyses that showed negative correlations of attractiveness with 2,5-dimethylpyrazine, 2-phenylethanol, and indole.

The hypothesis that 3-hydroxybutanone was responsible for the greater attractiveness of AMF strain plates was examined by linear regression of attractiveness indices versus headspace concentration of 3-hydroxybutanone in AMF strain plates. Although the purpose was to determine why AMF strain plates were more attractive than MFF strain plates, the initial analyses were done using only AMF strain plates because MFF strain plates did not produce 3-hydroxybutanone.

Correlations of attractiveness indices of AMF strain plates versus headspace concentration of 3-hydroxybutanone were positive, but not significant, for both males and females. Lack of significance does not invalidate the hypothesis, but indicates that differences in attractiveness of AMF plates from each other cannot be attributed unequivocally to differences in concentrations of 3-hydroxybutanone. When results for MFF strain plates and AMF strain plates were combined, the correlation was significant for both males and females (males: $r = 0.44$, $df = 28$ $P < 0.05$; females: $r = 0.51$, $df = 28$, $P < 0.01$). In this case, differences in concentrations of 3-hydroxybutanone are consistent with differences in attractiveness, but do not prove that 3-hydroxybutanone is responsible for the greater attractiveness of AMF strain plates compared with MFF strain plates.

Changes in the AMF Strain. We presented evidence that the AMF strain changed during the course of this work, at times producing volatiles that were considerably different from those produced by the MFF strain plates and at other times producing indole and other volatiles similar to those produced by MFF strain plates. Indications that attractiveness also changed as the volatiles changed were evident in responses of males (Table 2). We did not observe consistent changes in volatiles production or attractiveness of plates with length of incubation time from 1–9 days.

In addition to changes that occurred during the current work, we also observed differences in AMF strain plates compared to previous work with the same uricase (+) strain of *E. agglomerans* (Robacker and Lauzon, 2002). In our former work, attraction to AMF strain plates was about 8× greater than attraction to uninoculated plates, whereas in the present study, the ratio was only about 2×. Indole was not found in our previous work. We were unable to determine if amounts of any other chemicals were different from our previous work.

To attempt to understand variation of volatiles profiles, we examined more extensively the biochemical capabilities of our strains of *E. agglomerans*. We found that subpopulations (cultured and plated as described in the Methods section), hereafter referred to as minority or majority subpopulations (or phenotypes), existed within both AMF and MFF strains. These minority subpopulations were different in terms of their ability to utilize substrates, and their colonial morphologies. The AMF strain contained one majority and one minority phenotype. The MFF strain contained one majority and two minority phenotypes.

The majority AMF subpopulation completely utilized uric acid when grown on the uric acid medium. The medium maintained a peach color indicating that the medium was near neutral pH. This indicates that utilization of the uric acid was likely accompanied by production of acidic fermentation products. This is substantiated in part by our chemical results. The color of the medium remained constant for approximately 7 days, and then the medium became progressively more alkaline (pink) as the remaining uric acid was utilized and other constituents within the medium were spent.

The minority AMF subpopulation did not completely utilize uric acid when grown on uric acid medium under identical conditions and time. Instead, the subpopulation demonstrated only partial utilization of the uric acid; the medium was more alkaline and was pink in color. Acid by-products were either absent or in amounts that did not affect the pH of the medium enough to detect them visually.

The majority MFF subpopulation gave observable traits on the uric acid medium similar to those seen for the minority AMF subpopulation. The majority MFF subpopulation produced compounds that turned the UA medium a deep, bright pink color, and only partially degraded the uric acid. Therefore, acidic compounds, if produced, did not drive the pH of the medium down. This may be explained by the fact that the majority MFF subpopulation did not follow a butylene glycol fermentation route from pyruvate. The majority AMF subpopulation, however, did and likely produced acidic compounds that appreciably altered the pH of the medium toward acidity. This explains why the AMF strain produced 3-hydroxybutanone and this compound was not detected for the MFF strain. 3-Hydroxybutanone is an intermediate of butylene glycol fermentation.

The majority MFF subpopulation and the minority AMF subpopulation also produced indole from tryptophan degradation. The majority AMF subpopulation did not. The emergence of a minority subpopulation within the AMF strain that produces indole may explain why during our previous work the AMF strain was more attractive to MFF than during our current studies.

Two minority subpopulations were isolated from the majority MFF subpopulation. The majority colonial morphology resembled the majority AMF subpopulation morphology with one exception. The majority MFF subpopulation was rougher in colonial texture than the typical smooth colonies of the AMF subpopulation. Both colonial types were circular to irregular, translucent, and pigmented, pale-yellow. The minority colonial morphologies included a smooth yellow colony and a smooth, off-white colony. All three MFF subpopulations grown individually on uric acid medium gave different observable results. As mentioned earlier, the majority MFF subpopulation cleared the uric acid to some extent, and the medium turned deep pink in color. The smooth yellow MFF subpopulation utilized uric acid less than the majority subpopulation, and the medium turned to a color close to that of peach, a combination of yellow, orange, and pink. This reaction was

most similar to that associated with the majority AMF subpopulation. The smooth off-white colony did not appear to utilize uric acid, and the medium turned yellow. Equal combinations of all three subpopulations yielded partial utilization of uric acid and a yellow medium. Combination of the two minority subpopulations resulted in a peach-colored plate. Therefore, despite the fact that the majority MFF subpopulation alone gave a strong alkaline reaction on the UA plate, in mixed company, the end result was quite different.

These findings suggest that microbial population ecology is an important aspect of chemical ecology and insect behavior. The population shifts that we observed may have been a result of repeated subculture within the laboratory setting or may reflect polymorphisms specific to each fruit fly species. *Enterobacter agglomerans* has been isolated routinely from fruit flies in four genera of economically important Tephritidae, their host fruit, oviposition sites, and in their natural food. These commonalities are intriguing and additional biochemical and molecular examination of strains of *E. agglomerans* may provide important insight toward insect behavior and biology.

Effect of Uricase on Volatiles and Attractiveness. The title of a previous paper (Robacker and Lauzon, 2002) implied that the ability of *E. agglomerans* to metabolize uric acid affected volatiles production and attractiveness to MFF, although these conclusions were not stated in the paper. These ideas were based on findings that a uricase (+) strain, but not a uricase (−) strain, produced 3-hydroxybutanone, that the uricase (+) strain produced greater amounts of several other volatiles compared with the uricase (−) strain, and the uricase (+) strain was more attractive than the uricase (−) strain. However, in the current research, we showed that two uricase (+) strains differed just as much in volatiles production and attractiveness as did the uricase (+) and (−) strains in the previous work. Taking results of both studies into account, there is no reason to conclude that differences in volatiles production and attractiveness are tied to whether or not the strains can metabolize uric acid.

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GENERALIZATION OF A HABITUATED FEEDING DETERRENT RESPONSE TO UNRELATED ANTIFEEDANTS FOLLOWING PROLONGED EXPOSURE IN A GENERALIST HERBIVORE, *Trichoplusia ni*

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Abstract—The possibility of generalization of habituated response to unrelated feeding deterrents following prolonged exposure was examined in third instar *Trichoplusia ni* (Lepidoptera: Noctuidae) larvae by rearing them on antifeedants and then testing with other unrelated antifeedants. We introduced neonate larvae (<24-hr old) onto cabbage leaves treated with crude seed extracts of *Melia volkensii* (Meliaceae) or oil of *Origanum vulgare* ("oregano") (Lamiaceae) and allowed them to feed until early in the third instar. Naïve larvae were reared on cabbage leaves treated with carrier solvent alone. Both experienced and naïve larvae were tested for feeding deterrent response with the same and the different extracts in a leaf disc choice bioassay. Habituation was generalized to both *M. volkensii* and oregano following prolonged exposure to either plant extract and also to a pure allelochemical, thymol, following prolonged exposure to either digitoxin or xanthotoxin. However, there was no generalization of the habituated response to oregano following prolonged exposure to digitoxin or thymol, or to thymol or xanthotoxin following prolonged exposure to oregano or *M. volkensii*. Our results demonstrate that habituated response to feeding deterrents in a polyphagous insect herbivore can be generalized among and between plant extracts and pure allelochemicals, but not in all situations. The implications of such behavioral plasticity in herbivorous insects for the use of antifeedants as crop protectants or for host plant shifts is discussed.

Key Words—Feeding deterrent, *Melia volkensii*, *Origanum vulgare*, digitoxin, thymol, xanthotoxin, *Trichoplusia ni*.

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INTRODUCTION

Habituation refers to a decrease in response (which is not due to sensory adaptation or motor fatigue) as a result of repeated presentations of a single stimulus (Carew and Sahley, 1986). Habituation differs from sensory adaptation in its ability to be terminated or reversed immediately by a novel or noxious stimulus (Thompson and Spencer, 1966).

Decreased feeding deterrent response or possibly habituation to chemical deterrents is potentially a means of altering host selection behavior, enabling an insect to eat a previously unacceptable food (Bernays and Chapman, 1994) and is adaptive when it occurs at a nontoxic concentration (Glendinning and Gonzalez, 1995). There are many examples of gustatory habituation in phytophagous insects showing that the taste rejection response to deterrent compounds markedly decreases after several hours or days of repeated dietary exposure to the same compound. For example, decreased feeding deterrent response to azadirachtin has been reported for *Spodoptera litura* (Bomford and Isman, 1996), nicotine hydrogen tartrate in *Schistocerca gregaria* (Szentesi and Bernays, 1984), aristocholic acid in *Spodoptera frugiperda*, and caffeine in *Pseudaletia unipuncta* (Usher et al., 1988). We have demonstrated a decrease in feeding deterrent response following prolonged exposure to extracts of *Melia volkensii, M. azedarach*, oregano, and to the pure allelochemicals, thymol and xanthotoxin applied to cabbage leaves in *Trichoplusia ni* (Akhtar et al., 2003). Some specialist species such as *P. unipuncta*, *Plutella xylostella* (Plutellidae), and *Epilachna varivestis* (Coccinellidae) also exhibit a decrease in feeding deterrent response following prolonged exposure to a pure allelochemical, i.e., thymol applied to corn, cabbage, and bean plants, respectively (Akhtar and Isman, 2004b). In all of these studies, the feeding responses of insects were determined after exposing the insects to the same antifeedant being tested.

This raises the question of whether the same phenomenon occurs with unrelated compounds. This type of experience-based response has been described as "cross-habituation" (Huang and Renwick, 1995) or generalization of a habituated response (Glendinning, 1996; Glendinning et al., 1999, 2001, 2002). Although, it is not clear what causes the decrease in feeding deterrent response (for example, habituation, sensory adaptation, or motor fatigue), we restrict ourselves to the use of the term *generalization of habituated response* herein.

Generalization of habituated response to unrelated compounds following prolonged exposure has been reported previously. Blaney et al. (1986) reported a decrease in sensitivity of the feeding deterrent receptor in tobacco hornworms, *Manduca sexta*, exposed to a diet containing salicin for 2.5 days. Dietary exposure to salicin reduced sensitivity not only to that deterrent, but simultaneously to caffeine.

Huang and Renwick (1995) reported that *Pieris rapae* larvae accepted foliage of *Tropeolum majus* (nasturtium) following exposure to the unrelated feeding

deterrents strophanthidin, cymarin, erysimoside, digitoxigenin, cucurbitacin, and rutin. *T. majus* is not acceptable to *P. rapae* larvae as a host plant following feeding on cabbage foliage, due to the presence of chlorogenic acid, a feeding deterrent for *P. rapae*. Glendinning (1996) and Glendinning et al. (1999, 2001, 2002) reported that *M. sexta* larvae showed a decrease in feeding deterrent response to caffeine and salicin after 24 h or 48 hr of dietary exposure. This phenomenon of habituation was generalized to salicin with previous exposure to caffeine and *vice versa*, but not to aristolochic acid, *Grindelia* extract, or *Canna* extract, as indicated by the electrophysiological recording of bitter-sensitive taste cells of *M. sexta*. All these examples show a generalization of habituated response to unrelated compounds when reared on one compound and tested with another unrelated compound, or reared on a compound and tested with a plant extract.

In this study we extended this work by examining if a habituated response to one antifeedant generalizes to another unrelated antifeedant under different conditions: (1) reared on one plant extract and tested with another unrelated plant extract; (2) reared on a plant extract and tested with unrelated pure allelochemicals; (3) reared on a pure allelochemical and tested with an unrelated plant extract; and (4) reared on a pure allelochemical and tested with an unrelated pure allelochemical.

The plant extracts (*Melia volkensii* and oregano oil) and pure allelochemicals (digitoxin, thymol, xanthotoxin) are demonstrated feeding deterrents for *T. ni* larvae (Akhtar and Isman, 2004a), and the latter represent discrete chemical classes of antifeedants. Although *T. ni* is primarily known as a pest of cruciferous plants, it also attacks several other crops including celery, an important source of xanthotoxin. Thymol, digitoxin, *M. volkensii*, and oregano might not be expected to occur in the normal host-plant range of the test species but it may well encounter closely related phenolics or terpenoids.

Studies relating to generalization of a habituated response to unrelated compounds are considered very important (1) because of their potential relevance to phytophagous insects in the field; insects that have become habituated to a deterrent compound in one plant species might tolerate novel deterrent compounds in other plant species, (2) for understanding insect feeding behavior in relation to pest management strategies based on antifeedants (as prolonged exposure to one feeding deterrent could lead to tolerance of other unrelated antifeedants), and (3) in understanding the potential for host-plant shifts and range extension in herbivorous insects.

METHODS AND MATERIALS

Plant Material

Cabbage plants (*Brassica oleracea* var. Stonehead) were used for bioassays and to maintain the *T. ni* colony. Plants were grown in plastic pots containing a

mixture of sandy loam soil and peatmoss (4:1) in the greenhouse at the University of British Columbia, Vancouver, BC, Canada. Plants used in the bioassays were 5- to 6-wk-old.

Test Substances

Plant Extracts. A refined extract of the seeds of *M. volkensii* (Meliaceae) was obtained from the University of Nairobi, Kenya, and oil of oregano was provided by EcoSMART Technologies Inc. (Nashville, TN). *Melia volkensii* extract contains a number of limonoids, including volkensin (20.3%), salannin (13.5%), 1-cinnamoyltrichilinin (1%), melanin (1%), 1-tigloyltrichilinin (1.5%), 1-acetyltrichilinin (1%), ohchinin-3-acetate (6%), and meliacin (1%) (Rajab and Bentley, 1988). *M. volkensii* does not contain azadirachtin (the major antifeedant and inseticidal constituent of neem, *Azadirachta indica* seeds), but salannin and ohchinin-3-acetate are present in neem seed extracts.

Origanum vulgare (Lamiaceae), usually known as oregano, is a common culinary herb. Major compounds in our sample of oregano oil are carvacrol (70%) and *p*-cymene (14.7%) (R. Bradbury, unpublished data). Thymol is a minor constituent at 1.45%. We consider oregano and pure thymol as unrelated, as the quantity of thymol is very small in the oregano oil used in our experiments. Oregano is known for its antifungal (Thompson, 1989), antiviral, antibacterial (Sivropoulou et al., 1996), insecticidal, and strong antifeedant properties against a number of insects (Sivropoulou et al., 1996; Karpouhtsis et al., 1998; Isman et al., 2001).

Pure Allelochemicals. Xanthotoxin (99%), digitoxin (97%), and thymol (99.5%) (Figure 1) were purchased from Sigma Chemical Co. (St. Louis, MO.).

Xanthotoxin, a furanocoumarin, is a potent antifeedant for *Spodoptera litura* (Yajima and Munakata, 1979) and *S. exigua* (Berdegue, et al., 1997), and widely toxic to generalist insect herbivores (Berenbaum et al., 1991).

Digitoxin, a cardenolide, is produced commercially for therapeutic purposes from the foxglove, *Digitalis lanata* (Mastenbroek, 1985), or *D. purpurea* (Scrophulariaceae), and possesses toxic and antifeedant properties against insects (Sachdev-Gupta et al., 1993) and mammals (McKenzie and Dunster, 1986).

Thymol, a monoterpenoid phenol, and a major constituent of garden thyme, *Thymus vulgaris* (Lamiaceae), possesses toxic and antifeedant properties against *S. litura* (Hummelbrunner and Isman, 2001).

Test Insects. Trichoplusia ni (Lepidoptera: Noctuidae) were obtained from an established laboratory colony maintained for >50 generations. Larvae of *T. ni* were reared on an artificial diet (No. 9795, Bioserv Inc., Frenchtown, NJ), supplemented with finely ground alfalfa (to improve acceptability), and vitamins (No. 8045, Bioserv Inc.).

Solvents. Methanol (MeOH) or methanol/dichloromethane (2:1, v/v) was used as carrier.

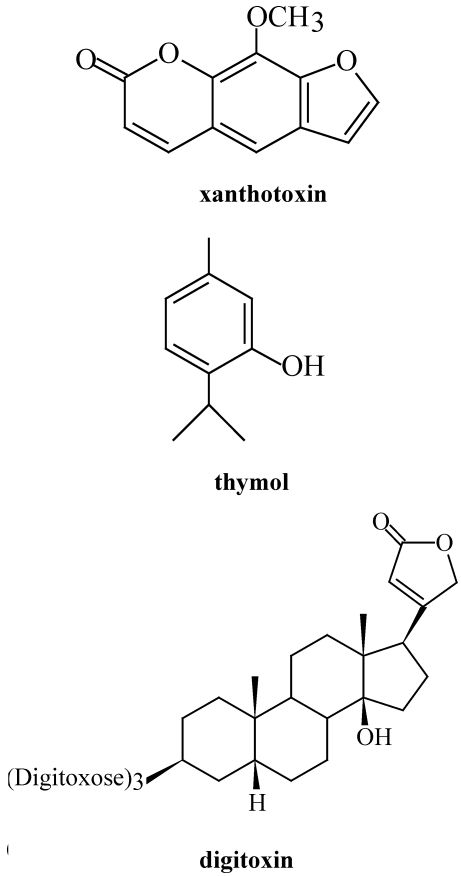

FIG. 1. Chemical structures of pure allelochemicals.

General Procedure

Training. There were two groups of larvae for each experiment, experienced and naïve. The experienced group was exposed to the plant extract or pure allelochemical from the neonate stage until tested as third instars. A 1-ml solution of the plant extract or pure allelochemical was applied to a cabbage leaf (approximately 100–110 cm^2) using a micropipette (0.5 ml on each side of the leaf). Concentrations used for rearing were determined in preliminary bioassays and did not cause any observable growth inhibition or toxicity to the insects. For all test substances, concentrations used in bioassays were expected to cause 60–75% feeding deterrence, on the basis of our previous investigation (Akhtar and Isman, 2004a). The chamber for training was a transparent plastic cylinder (220 × 300 mm) (diam × ht).

The cut leaf petiole end was placed into a 100-ml plastic cup filled with water, in the center of the chamber. The cylinder was covered with a clear plastic lid to prevent larvae from escaping. Approximately 20 neonate larvae (<24-hr-old) were placed on each cabbage leaf and allowed to feed *ad libitum* until bioassays were conducted. New leaves were introduced every 2nd day or as needed. The naïve group was reared for the same length of time and under the same conditions on cabbage foliage treated only with MeOH or MeOH/dichloro methane (carrier controls).

Testing. After the training period, larvae from the experienced and the naïve groups were tested in leaf disc choice bioassays (Isman et al., 1990; Akhtar et al., 2003) to determine their feeding responses to unrelated antifeedants (plant extracts or pure allelochemicals).

Leaf Disc Choice Bioassay. Larvae were starved 4–5 hr prior to each bioassay. Fresh leaf discs were cut from greenhouse-grown cabbage ~5-wk-old, using a #6 (1.2 cm diam, 1.13 cm^2 area) cork borer. Control leaf discs were painted on each side with 7.9 μl of the carrier solvent and test leaf discs with the same amount of the test substance. After the solvent dried, one treated and one control disc were placed in each compartment [4.2 × 3.0 cm ($l \times w$)] of a plastic assay tray with a small piece of moistened cotton to prevent desiccation. The distance between the two discs was approximately 0.7 cm. After the starvation period, one larva was introduced gently into the center of each compartment with forceps and allowed to feed. The trays were covered with plastic lids. The plastic trays with larvae and test discs were put into a clear plastic box [(39 × 27 × 14 cm ($l \times w \times h$)] lined with moistened paper towel, and the box was placed in an illuminated growth chamber at 26°C. When approximately 50% of the control leaf discs had been eaten (normally 3–5 hr), larvae were removed from the trays. The leaf discs were placed on glass plates, and a digital picture was obtained by using an IS-500 digital imaging system (Alpha Innotech Corp.). Areas of control and treated leaf discs eaten were measured by using Scion Image software.

A feeding deterrence index (FDI) was calculated using the formula

$$\text{FDI} = 100\{(C - T)/(C + T)\}$$

where C and T are the control and treated leaf areas consumed by the insect.

Reared on a Plant Extract and Tested with Both the Same and a Different Extract. This experiment sought to determine whether a habituated response to a plant extract generalized to an unrelated plant extract. The objective was to measure the difference in the feeding response of the two experienced groups under similar testing conditions (one tested with the same plant extract and the other with an unrelated plant extract) with the naïve group ($N = 40$/group).

Experienced groups were reared on *M. volkensii* at 0.01 mg/cabbage leaf and oregano at 2.5 mg/cabbage leaf until the third instar. Experienced and naïve groups were tested with oregano extract (80 μg/cm^2) and *M. volkensii* extract (15 μg/cm^2).

Reared on a Plant Extract and Tested with Pure Allelochemicals. This experiment sought to determine whether the habituated response following prolonged exposure to a plant extract generalized to unrelated pure allelochemicals. The experienced groups were reared on *M. volkensii* (0.01 mg/cabbage leaf) or oregano (2.5 mg/cabbage leaf) until the third instar. Naive groups were reared on cabbage leaves treated with methanol. Experienced and naïve groups were tested with thymol (40 μg/cm^2) or xanthotoxin (0.6 μg/cm^2) ($N = 25$/group).

Reared on Pure Allelochemicals and Tested with a Plant Extract. This experiment sought to determine whether the habituated response following prolonged exposure to pure allelochemicals generalized to unrelated plant extracts. The experienced groups were reared on digitoxin or thymol at 0.01 and 1.0 mg/cabbage leaf, respectively, until the third instar. Naive groups were reared on cabbage leaves treated with MeOH:DCM (2:1, v/v). The experienced and naive groups were tested with oregano oil (80 μg/cm^2) ($N = 23$/group).

Reared on a Pure Allelochemical and Tested with Another Pure Allelochemical. This experiment sought to determine whether the habituated response following prolonged exposure to pure allelochemicals generalized to unrelated pure allelochemicals. The experienced groups were reared on digitoxin or xanthotoxin at 0.01 mg/cabbage leaf until the third instar. Naive groups were reared on cabbage leaves treated with MeOH:DCM (2:1, v/v). The experienced and the naive groups were tested with thymol (40 μg/cm^2) ($N = 25$/group).

Data Analysis. Data were analyzed on the basis of actual numbers observed (variance of the sample means were determined to be homogeneous) by analysis of variance (ANOVA) (Zar, 1984) using statistics software (Statistix 7, 2000). Where significant F values were found, Tukey's HSD test was used to test for significant differences between individual treatments. The alpha level used was 0.05.

RESULTS

Reared on a Plant Extract and Tested with Both the Same and the Different Extracts. A two-way ANOVA (treatment $\times$ group) on the feeding deterrence indices of third instar *T. ni* larvae showed that there was no significant main effect of treatment (*M. volkensii* and oregano). There was a main effect of group [$F(2, 234) = 18.10$, $P < 0.001$] with experienced larvae showing a greater decrease in feeding deterrent response than naïve larvae. There was no significant (treatment $\times$ group) interaction. No differences were found between the deterrent responses of larvae reared on *M. volkensii* or oregano and tested with the same or with the opposite extract, but all differed from their respective naïve groups (Tukey's test, $P < 0.05$) (Figure 2).

Reared on a Plant Extract and Tested with Pure Allelochemicals. A two-way ANOVA (treatment $\times$ group) on the feeding deterrence indices of experienced

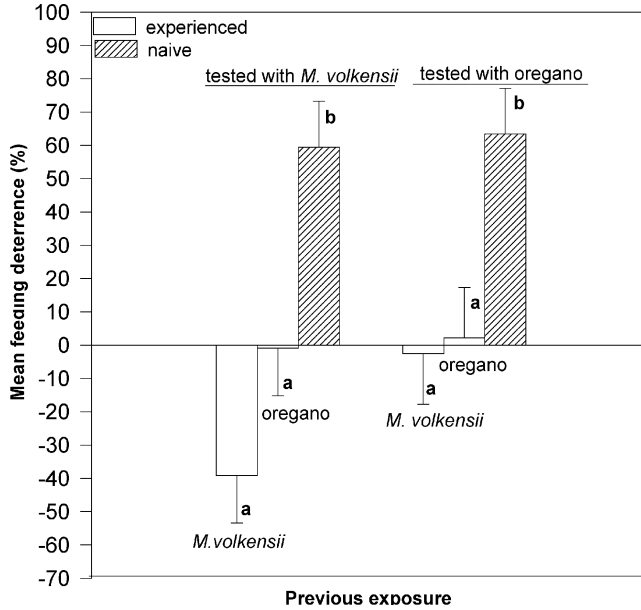

FIG. 2. Feeding responses of third instar *T. ni* to *M. volkensii* and oregano (represented by bars) following prolonged exposure to *M. volkensii* or oregano. Feeding deterrence means (±SE) followed by the same letter do not differ significantly (Tukey's test, $P < 0.05$, $N = 40$/group).

(reared on *M. volkensii* at 0.01 mg/cabbage leaf andoregano at 2.5 mg/cabbage leaf) larvae to thymol or xanthotoxin and naïve groups (Figure 3) did not show a significant main effect of treatment [$F(3, 192) = 0.7$, $P = 0.72$]. In addition, there was no main effect of group [$F(1, 192) = 0.39$, $P = 0.53$], with experienced and naïve larvae showing the same feeding deterrent response to thymol or xanthotoxin. There was no (treatment × group) interaction [$F(3, 192) = 0.65$, $P = 0.58$], with larvae reared on *M. volkensii* and oregano showing the same feeding deterrent response to thymol and xanthotoxin as the naïve larvae (Figure 3).

Reared on Pure Allelochemicals and Tested with a Plant Extract. A one-way ANOVA of the feeding deterrence indices of experienced groups (reared on digitoxin and thymol) and the naïve group to oregano oil did not produce a significant F value [$F(2, 66) = 0.01, P = 0.99$] (Figure 4) showing that prolonged exposure to the pure allelochemicals had no effect on feeding deterrent responses of larvae to oregano oil.

Reared on a Pure Allelochemical and Tested with Another Pure Allelochemical. A one-way ANOVA of the feeding deterrence indices of experienced groups

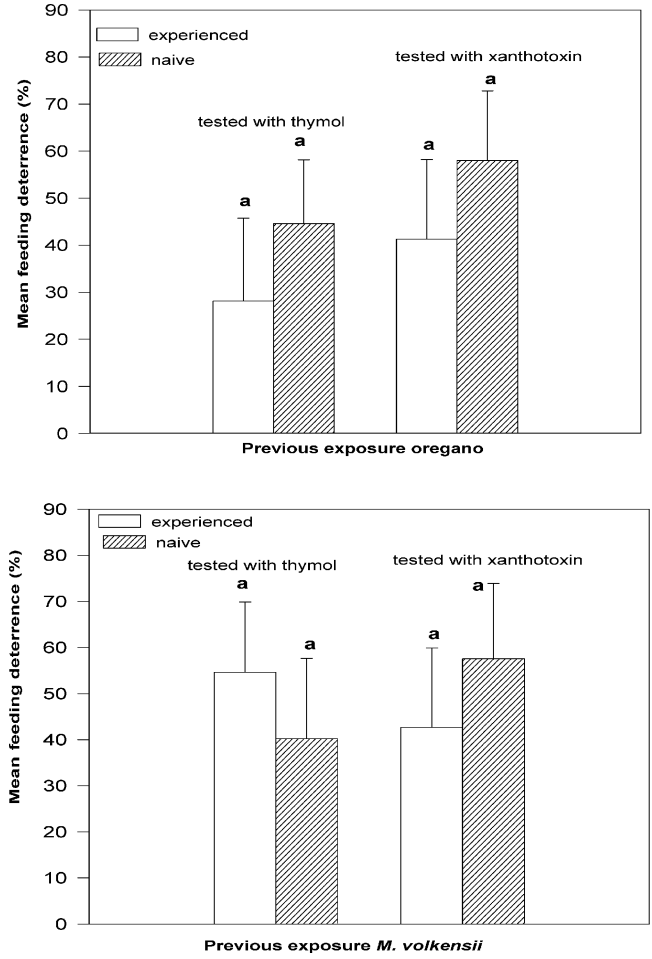

FIG. 3. Feeding responses of experienced (reared on *M. volkensii* or oregano) and naïve third instar *T. ni* larvae to thymol and xanthotoxin. Mean feeding deterrence (represented by bars) (±SE) followed by the same letter do not differ significantly (Tukey's test, $P < 0.05$, $N = 25$/group).

(reared on digitoxin and xanthotoxin) and a naïve group to thymol produced a significant F value [$F(2, 72) = 4.51$, $P = 0.01$) (Figure 5). Comparison of means of feeding deterrence indices of experienced and naïve groups showed that both experienced groups (reared on digitoxin or xanthotoxin) demonstrated a decrease in feeding deterrent response to thymol unlike the naïve group (Tukey's test, $P < 0.05$).

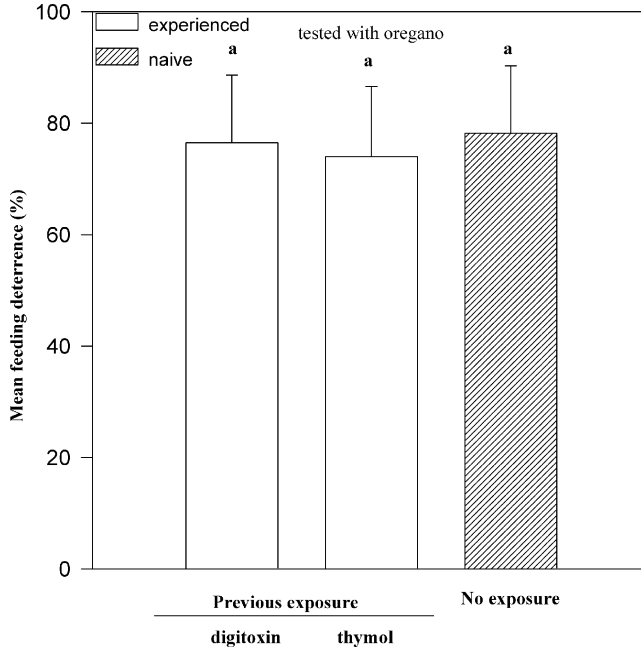

FIG. 4. Feeding response of third instar *T. ni* larvae to oregano (represented by bars) following prolonged exposure to digitoxin and thymol. Means (±SE) followed by same letters do not differ significantly (Tukey's test, $P < 0.05$, $N = 23$/group).

DISCUSSION

The results indicate that third instar *T. ni* larvae showed a generalization of habituated response to antifeedants following prolonged exposure, although not in all cases. *T. ni* larvae showed a generalization of habituated response to a plant extract and a pure allelochemical with previous exposure to an unrelated plant extract and pure allelochemicals, respectively. Mechanisms accounting for decreased responsiveness to feeding deterrents following prior exposure could include gustatory effects—peripherally in the chemosensilla or at the level of neural integration, physiological effects—induction of detoxicative enzymes in the gut (Snyder and Glendinning, 1996), or some combination of these. We contend that our present results constitute habituation because we have previously demonstrated this phenomenon with a number of plant extracts and compounds in *T. ni*, and more importantly, shown that larvae can be dishabituated through introduction of a novel noxious stimulus between the training and test periods (Akhtar et al., 2003).

There was a generalization of habituated response to oregano in larvae with previous exposure to *M. volkensii* extract and *vice versa*. This generalization to

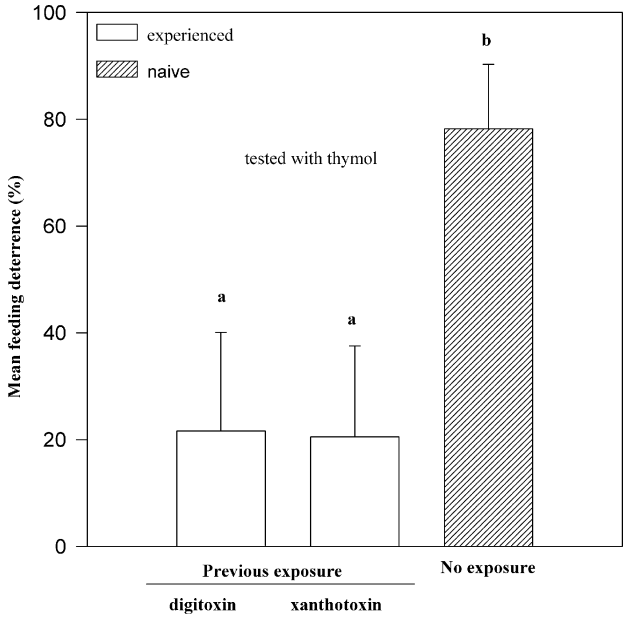

FIG. 5. Feeding response of third instar *T. ni* larvae to thymol (represented by bars) following prolonged exposure to digitoxin and xanthotoxin. Means (±SE) followed by same letters do not differ significantly (Tukey's test, $P < 0.05$, $N = 25$/group).

unrelated plant extracts was not shown by naïve larvae in either case. A similar generalization of habituated response was also exhibited by *T. ni* larvae to thymol following prolonged exposure to the unrelated digitoxin or xanthotoxin unlike the naïve group.

There is a considerable amount of evidence relating a habituated response to antifeedants following prolonged exposure, to changes in receptor characteristics, measured by a change in firing characteristics of gustatory sensilla (Blaney et al., 1986). Dietary exposure to salicin for 2.5 days in tobacco hornworms, *Manduca sexta*, reduced sensitivity not only to that deterrent, but simultaneously to caffeine, despite the fact that the latter compound probably acted on a different receptor site on the same deterrent cell (Blaney et al., 1986). A similar mechanism might explain the observed generalization of habituated response in *T. ni* to unrelated feeding deterrents following prolonged exposure.

The generalization of habituated response observed might be explained on the basis that a common transduction pathway is shared by the plant extracts or pure allelochemicals (Glendinning et al., 1999) within the same bitter-sensitive taste cells. It could be that they elicit excitatory responses that are virtually identical in terms of maximal firing, rate and temporal pattern of firing, resulting in a lack

of discrimination by the central nervous system as was the case with salicin and caffeine in *M. sexta* (Glendinning et al., 1999, 2002). Electrophysiological studies are needed to confirm this.

Our experiments did not show a significant generalization of habituated response when *T. ni* larvae were reared on a plant extract (oregano) and tested with pure allelochemicals (thymol or xanthotoxin) or reared on pure allelochemicals (digitoxin or thymol) and tested with a plant extract (oregano). If pure allelochemicals and plant extracts are sensed by different populations of bitter-sensitive taste cells, they would produce a distinct spatial pattern of activation within the primary projection site of the bitter-sensitive taste cells, i.e., the subesophageal ganglion (SOG). If habituated response is assumed to be restricted to loci in SOG that receive input from taste cells responsive to pure allelochemicals, then it would not be expected to generalize to loci in SOG that receive input from taste cells responsive to plant extracts (mixtures) or *vice versa* (Glendinning et al., 2002).

Glendinning (1996) and Glendinning et al. (1999, 2001, 2002) reported that the habituated response to salicin generalized to caffeine and *vice versa*, in *M. sexta* larvae, but not to aristolochic acid, *Grindelia* extract, or *Canna* extract, as indicated by the electrophysiological recording of bitter-sensitive taste cells of *M. sexta* even though salicin and aristolochic acid stimulate the same bitter-sensitive taste cells. The lack of generalization of habituated responses by *T. ni* in some situations (e.g., when reared on a plant extract and tested with a pure compound, or *vice versa*) in our experiments suggests that some central phenomena may also be involved in this process, apart from changes in receptor characteristics (R. F. Chapman, personal communication 2003).

In summary, our experiments have shown that *T. ni* larvae are capable of showing a generalization of the habituated response in a number of situations. Decreased feeding deterrent response to related or unrelated feeding deterrents following prolonged exposure could limit the practical application of such natural compounds for pest control. Information obtained from the present and previous studies (Akhtar et al., 2003; Akhtar and Isman, 2004a) on feeding behavior in larvae of *T. ni* suggests that these insects are likely capable of exploiting food resources far beyond their normal host-plant range.

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DIFFERENTIAL ACTIVITY OF PEROXIDASE ISOZYMES IN RESPONSE TO WOUNDING, GYPSY MOTH, AND PLANT HORMONES IN NORTHERN RED OAK (*Quercus rubra* L.)

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Abstract—We measured total peroxidase activity and the activities of peroxidase isoforms in leaves of red oak (*Quercus rubra* L.) seedlings exposed to wounding and plant hormones in the greenhouse. Activity of specific peroxidase isoforms was induced differentially by gypsy moth wounding, mechanical wounding, and the wound-associated plant hormone jasmonic acid. Activity of one isoform was enhanced modestly by treatment with salicylate. A study of peroxidase activity in naturally occurring galls elicited on red oak leaves by 12 hymenopteran and dipteran insect species found 16 POD isoforms, 11 of which were differentially induced or suppressed in galls compared with leaves. In both studies, total peroxidase activity as measured spectrophotometrically was not clearly related to activity of these isoforms. These results indicate that red oak seedlings and trees may respond specifically to wounding, particular insects, and plant signals through changes in the activities of individual isozymes.

Key Words—Peroxidase, isozyme, PR protein, *Quercus rubra*, wounding, gypsy moth, jasmonic acid, salicylic acid, galls.

INTRODUCTION

Plant peroxidases (E.C. 1.11.1.7) are heme-containing enzymes whose primary function is to oxidize a variety of hydrogen donors at the expense of hydrogen peroxide. Peroxidase (POD) activity has been correlated with a wide range of

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plant physiological processes, including lignification, suberization, somatic embryogenesis, auxin metabolism, wounding, and disease resistance (Ye et al., 1990; Zimmerlin et al., 1994). PODs are ubiquitous enzymes in plants, often occurring as multiple isoforms; for example, they are encoded by 73 different genes in *Arabidopsis thaliana* (Duroux and Welinder, 2003). Such an abundance of isoforms is consistent with diverse physiological functions for the peroxidase family (Siegel, 1993). POD polymorphisms are often used in taxonomic and population studies.

Plants respond to various environmental challenges with diverse biochemical changes. For example, they commonly elevate concentrations of secondary compounds in response to pathogen infection or insect herbivory (Karban and Baldwin, 1997). Other plant responses include lignification (Vance et al., 1980; Díaz and Merino, 1998) and the production of pathogenesis-related (PR) proteins (Van Loon, 1985). PODs comprise one important class of PR proteins (PR-9) implicated in these "defense responses," in which an important role is to catalyze the formation of phenolic radicals at the expense of H_2O_2 (Gaspar et al., 1985). PODs may also oxidize phenolic monomers to form lignin (Siegel, 1954; Mäder et al., 1980; Grisebach, 1981), function in H_2O_2 production (Elstner and Heupel, 1976; Mäder et al., 1980), and metabolize indole acetic acid (Endo, 1968; Mato et al., 1988). Each plant species typically displays a unique pattern and number of soluble and wall-bound isozymes that may respond differentially to environmental stimuli. Some of the factors known to influence POD isozyme expression are mechanical wounding (Birecka and Miller, 1974; Svalheim and Robertsen, 1990), mite feeding (Bronner et al., 1991), pathogen infection (Lagrimini and Rothstein, 1987; Ye et al., 1990), plant hormones (Ridge and Osborne, 1970; Birecka and Miller, 1974; Neuman et al., 1992), and plant developmental stage (Pao and Morgan, 1988; Biles and Martyn, 1993).

Although POD functions have been well studied in tobacco, horseradish, and other herbaceous species (Birecka and Miller, 1974; Lagrimini and Rothstein, 1987; Kawaoka et al., 1994), activity studies in woody plants are uncommon (Goodin et al., 1993; Dowd et al., 1998a). Since insect attack elicits changes in leaf polyphenols in some oak species (e.g., Rossiter et al., 1988), it is likely that the activities of oak phenolic-oxidizing enzymes and isozymes also change in response to wounding. No studies have examined the enzymatic responses of oak species to herbivores and pathogens, or the role of chemical signaling molecules likely to be involved in these responses.

Responses to herbivores, pathogens, and other environmental stimuli are generally regulated by a network of signal transduction pathways in which jasmonic acid (JA) and salicylic acid (SA) are key signaling molecules (Glazebrook, 2001; Thomma et al., 2001; Kunkel and Brooks, 2002). Wounding and herbivore damage cause rapid increases in JA (Bostock, 1999; Reymond et al., 2000), triggering systemic defenses against herbivores and necrotrophic pathogens. Infection by

biotrophic pathogens can elicit rapid increases in SA (Gaffney et al., 1993; Ryals et al., 1994) and systemic expression of defenses against a range of pathogens.

We assayed POD activity in northern red oak (*Quercus rubra* L.) leaf tissue subsequent to mechanical wounding, wounding by gypsy moth (*Lymantria dispar* L.; Lymantriidae) larvae, and treatment with exogenous JA and SA. We also examined POD activity in galls formed on red oak leaves in response to 12 naturally-occurring insect species. Because red oak produces diverse phenolic substrates (Li and Hsiao, 1975), and because plants are known to express substrate-specific POD isozymes (Calderón et al., 1990), we separated oak PODs by gel electrophoresis and isoelectric focusing and examined elicitation or suppression of individual isoform activities in relation to total POD activity.

METHODS AND MATERIALS

Seedling Oak Growth. Red oak (*Quercus rubra* L.) half-sib seedlings were germinated from acorns collected from a single tree on the Penn State University Park campus in flats containing Metro-Mix 250®growth medium in late June in a glasshouse at 20°C. One week after germination, seedlings were transplanted to 20 × 42-cm pots containing wetted Metro-Mix 250 and 14-14-14 (N-P-K) Osmocote®slow-release fertilizer incorporated at a rate of 550 ml fertilizer beads per 0.085 m^3 of growth medium. Seedlings were watered daily with tap water for 15 min at 7.5 l/hr, and supplemental lighting of 200–350 $\mu E/m^2$ was provided for 13 hr each day.

Treatments. Ten d after transplant, 102 4-wk-old seedlings (each approximately 15 cm in height) were randomly assigned to 1 of 7 treatment or control groups. One group of seedlings served as a control for any POD isozyme induction due to leaf removal or mechanical disturbance. Sample sizes for each treatment and controls ranged from 12 to 15 individual trees. At this age, red oak seedlings have produced one set of 4–5 leaves, all within a day. Leaves were chosen randomly for treatment and/or harvest.

Wounding was accomplished with gypsy moth larvae or mechanically with a hole punch. One to three starved fourth instar gypsy moth larvae were confined manually to a single leaf for the first 12 hr of treatment or until 20–30% of the leaf area was eaten. After removing and flash freezing this leaf, one or more larvae were left on the seedling to continue the insect wounding treatment for 1 wk. Larvae were added or removed over the 1 wk period to accomplish 30–50% leaf area removal. Mechanical wounding with a sterile 6-mm diam hole punch was carried out to mimic insect leaf removal for both the 12-hr and week-long wounding treatments. To prevent the movement of gypsy moth larvae between seedlings, squares of aluminum foil coated in Tanglefoot®were placed around the bases of all 102 seedlings.

Another group of seedlings was sprayed daily (0800 hr) with 5-mM JA in 4% EtOH, or a control solution without JA. Seedlings were removed from the main greenhouse room and sprayed until the JA or JA control solution dripped off the leaves, and allowed to dry for 30 min before being placed back in the main room.

Similarly, additional seedlings were sprayed at the same times with 5-mM SA, consisting of 173-mg SA in 250 ml of 0.005% Triton X-100, or a control solution without SA. The spraying procedure was identical to that of the JA treatment. Both spray regimes were chosen to be consistent with other studies assessing the impact of these signals on enzymatic and metabolic activity and transcript abundance of defense-related genes (e.g., Thaler et al., 1996; Zhang and Baldwin, 1997; Cipollini and Redman, 1999; Moore et al., 2003).

All unsprayed seedlings were jostled and tapped gently on the leaves for a few seconds to account for any seedling response to mechanical disturbance during the JA, SA, or control spraying.

Sampling and Extraction for Enzyme Analysis. Preliminary studies indicated that accumulation of insect-induced polyphenols could be detected after 7–10 d. Therefore, to capture any coordinated changes in POD levels a single treated leaf per seedling was removed at the base of the petiole with scissors 12 hr, 1 wk, and 2 wk (1 wk after the end of treatments) after the start of each treatment; these were flash-frozen in liquid N_2 and stored at -80°C until analysis. Frozen leaf tissue was homogenized in ice-cold extraction buffer with a chilled mortar and pestle at a buffer:tissue ratio of 20:1 (v:w). The extraction buffer contained 0.1-M potassium phosphate, pH 7.0, and 9% (w/v) polyvinylpolypyrrolidone. To break up cellular membranes, 10% (v/v) Triton X-100 was added to the extraction buffer at a rate of 1.6 μl/mg tissue (Jansen et al., 2001). Initially, extracts were centrifuged at 1100 $\times$ g for 15 min at 4°C; however, the viscosity of extracts from older leaves hindered adequate separation of the supernatant and pellet. Thus, samples from older leaves were centrifuged at 15000 $\times$ g for 15 min at 4°C. Densitometric measurements of POD isozyme bands from the same leaf extract exposed to each of the two centrifugation regimes indicated no major differences. Crude extract aliquots were placed into 0.5-ml Eppendorf tubes and frozen at -20°C for later protein assays or used immediately for POD isozyme analysis. These procedures are likely to have extracted primarily soluble PODs. All chemicals were purchased from Sigma Chemical Company, St. Louis, MO, or Bio-Rad Laboratories, Hercules, CA.

Total Peroxidase and Protein Assays. The total soluble POD specific activities of each oak tissue extract were determined by using a microplate reader. To measure total soluble POD, three replicates of 4-μl crude extract were each combined with 196-μl substrate, containing 10-mM guaiacol and 4-mM H_2O_2 in 0.1-M potassium acetate buffer, pH 4.5. The samples were shaken for 10 sec and read at 470 nm for 3 min, and the linear ΔOD/min/mg protein was used as a measure of relative POD activity. Protein content was determined (3$\times$) using the Bio-Rad Detergent-Compatible Protein Assay according to the instructions of the manufacturer.

Isoelectric Focusing. Soluble peroxidase isozymes were separated by using polyacrylamide gel electrophoresis – isoelectric focusing (IEF) with a Model 111 mini-IEF unit (Bio-Rad, Hercules, CA, USA). Gels were cast according to the manufacturer's instructions by using the following components: 50-μl 3/10, 200-μl 8/10, and 700-μl 3/5 ampholyte, 5.05-ml H_2O, 2.0-ml 24.25% acrylamide with 0.75% bis-acrylamide (w/v), and 2.0-ml 25% glycerol (v/v). After moderate degassing, 50 μl of 10% ammonium persulfate (w/v) and 5-μl TEMED were added to initiate polymerization. Preliminary studies indicated that this mixture of components resulted in the least difficulty in casting the gels and best separation of isozymes, which tended to be very acidic or very basic. Standards used were as described in the manufacturer's instructions (Bio-Rad, Hercules, CA, USA).

One μl of crude extract was added to each sample well with a Hamilton syringe and allowed to diffuse into the gel for 5 min. Isozymes were separated by stepwise increases in voltage of 100, 200, and 450 V for 15, 15, and 45 min, respectively. Following focusing, gels were soaked in 0.1-M potassium succinate (KSuc), pH 5.5, for 10 min to equilibrate the pH throughout the gel. Peroxidase isozymes were visualized with *o*-dianisidine and H_2O_2 in 0.1-M KSuc, pH 5.5, for 30 min, which was found to be more sensitive and reliable than guaiacol for visualizing POD in gels. The substrate was made by dissolving 8 mg/ml *o*-dianisidine in methanol, combining 2.5 ml of this solution with 97.5-ml KSuc buffer, and adding 44.8-μl 30% H_2O_2.

Immediately after visualization, gels were rinsed 4–6 $\times$ in KSuc buffer, and relative isozyme activities were quantified with a Shimadzu CS-9000U dual-wavelength scanning densitometer. After identifying the linear density range by using a dilution series, each gel lane was scanned at 470 nm (analytical) and 570 nm (reference) with a 0.4 $\times$ 5.0-mm beam. The relative activity of each isozyme was recorded as an integrated absorbance peak at a specific coordinate on the gel lane. Activities are expressed per mg protein. Isozyme bands were compared among different gels and gel lanes by determining their distance relative to a prominent anionic band hereafter called A4.4 and the bottom edge of the 1-cm gel. Isoforms are designated as cationic (C) or anionic (A) and by their isoelectric points (see Figure 1 for naming scheme).

Field Study of Gall PODs. While many galling insects chew host plant tissues, many are thought to suppress or modify normal plant responses to attack (Hartley and Lawton, 1992). We investigated whether such insects would influence POD activities in a manner similar to gypsy moth or artificial wounding. On May 22 and June 3, 1998, galls on red oak leaves formed by the gall wasps (Hymenptera: Cynipidae) *Amphibolips confluens* Harr., *A. inanis* Harr., *A. nubilipennis* Harr., *Andricus pallustris* O.S., *Callirhytis modesta* O.S., *C. pigra* O.S., *and C. tumificus* O.S., and by the gall midges (Diptera: Cecidomyiidae) *Macrodiplosis* spp. *(M. erubescens* O.S., *M. majalis* O.S., *M. niveipila* O.S.), *Polystepha americana* Felt, and an unknown cecidomyiid species were collected from

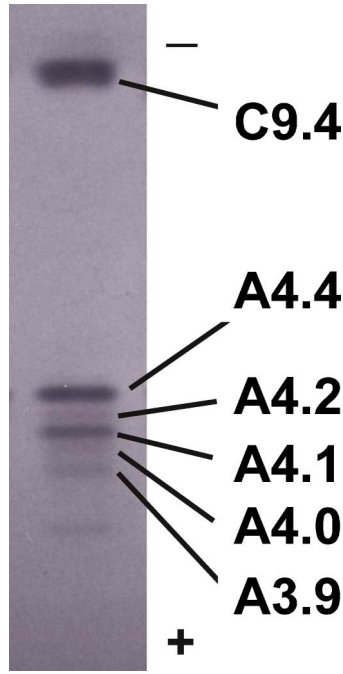

FIG. 1. Example isoelectric focusing gel showing the six most reliably-visualized POD isoforms in greenhouse-grown red oak leaves. Cationic: +, anionic: −.

21 trees in an oak-dominated forest in central Pennsylvania, USA, for peroxidase isozyme analysis. Galls ranged in age from 7 to 15 d; most age differences were between, not within, species. Trees ranged in size from 2 to 25 m, and at least 5 galls were collected from each tree at heights below 3 m. With each gall collected, a nearby ungalled, undamaged leaf of the same position and developmental stage on the same tree was removed at the base of the petiole for comparison. Insects were removed, and all tissues were immediately flash frozen in liquid N_2, held on dry ice, and stored at -20°C until identification and analysis as described above.

Statistical Analyses. Treatment and date effects on isozyme activity, total POD activity, and protein concentration were subjected to a repeated measures analysis of variance by using the mixed procedure of the SAS statistical package (SAS Institute, 1999). A "contrast" statement within the procedure was used to distinguish treatment effects significant at the $\alpha = 0.05$ level. Tukey's studentized range test was used to compare treatment and control means within each date. Paired t tests were used to determine significant differences in isozyme activity and protein content between field-collected red oak galls and leaves.

RESULTS

Total Seedling Protein and Peroxidase. Total protein concentration did not differ among the treatments but dropped slightly after 1 wk (Figure 2). Similarly, total soluble POD activity decreased after 1 wk and then recovered to or exceeded 12 hr levels after 2 wk (Figure 2). Specific contrasts indicated that total POD activity was less in the JA treatment and greater in the SA treatment than in both wounding treatments and the unwounded controls (Figure 2). A lack of difference between JA and SA solvent controls and wound treatments indicates that these were not solvent effects (Figure 2).

Seedling Isozymes. At least 10 POD isozymes were observed in the tissue extracts from red oak seedlings, but only six could be visualized reliably enough within treatments to be considered for statistical analysis (Figure 1). Of the six, one was strongly basic (pI > 9), while the other 5 were strongly acidic (pI < 4.5).

JA treatment and to a lesser extent GM wounding ($P = 0.08$) increased the activity of isozyme A4.1, but there were no significant treatment effects on the cationic isozyme C9.4 or the anionic isozymes A4.2 and A4.0 (Figure 3). All of these isozymes changed over time; activities of C9.4 and A4.1 increased, while activities of A4.2 and A4.0 declined over 2 wk (Figure 3).

Specific contrasts with controls indicated that isozyme A4.4 activity was significantly increased by gypsy moth wounding, mechanical wounding, and JA application, especially after 1 wk (Figure 3). JA treatment caused the greatest response, followed by mechanical wounding and GM wounding. While SA-treated seedlings contained more isozyme A4.4 activity than did untreated controls, SA-treated seedlings did not differ from SA controls, and SA controls (solvent treated) did not differ from untreated controls. The activity of A4.4 increased significantly with time (Figure 3).

The effects of both treatment and date were significant for isozyme A3.9 (Figure 3). Isozyme activity increased strongly over the course of the experiment, and was significantly increased by both gypsy moth and mechanical wounding, but not by JA (Figure 3). Over the entire experiment, there was a marginally significant effect of SA ($P = 0.06$).

Field Gall POD Study. Naturally-occurring galls and leaves exhibited 16 separable isoforms frequently enough for statistical analysis (Table 1). However, not all 21 sampled trees exhibited activity of all 16 isoforms; as few as 8 isoforms could be found in some individuals (data not shown). All galls generally contained significantly enhanced activity (compared with leaves) of cationic isoform C9.4, and significantly reduced activities of cationic isoform C8.2 and anionic isoforms A4.6, A4.4, A4.15, A4.1, A3.7, and A3.9 (Table 2) compared with control leaves on the same trees. Two additional anionic isoforms were suppressed at significance levels $0.10 > P > 0.05$.

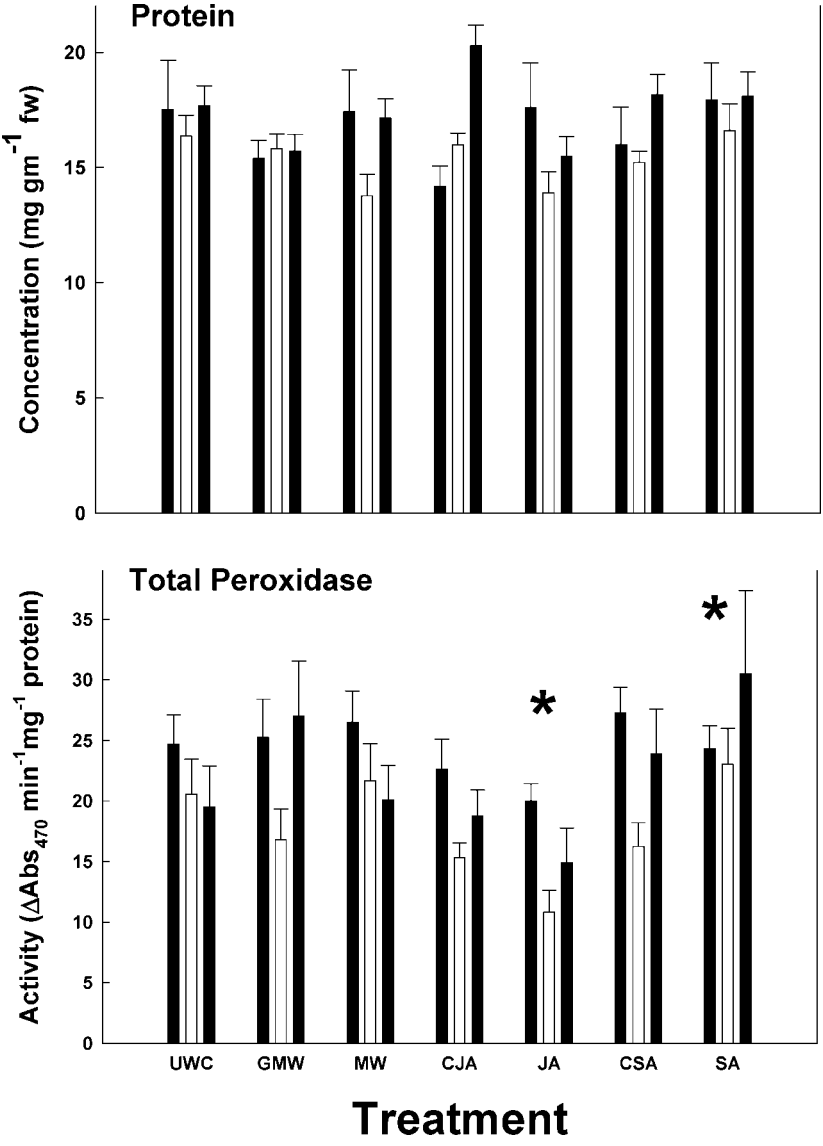

FIG. 2. Means (SE) of relative protein content (top) and total specific POD activity (bottom) of greenhouse-grown red oak leaves after 12 hr, 1 wk, and 2 wk (left to right in each trio of bars) of the following treatments: UWC—unwounded controls, GMW—gypsy moth wounding, MW—mechanical wounding, CJA—jasmonic acid solvent control, JA—jasmonic acid appliction, CSA—salicylic acid solvent control, SA—salicylic acid application. Asterisks indicate significant treatment effects ($P < 0.05$).

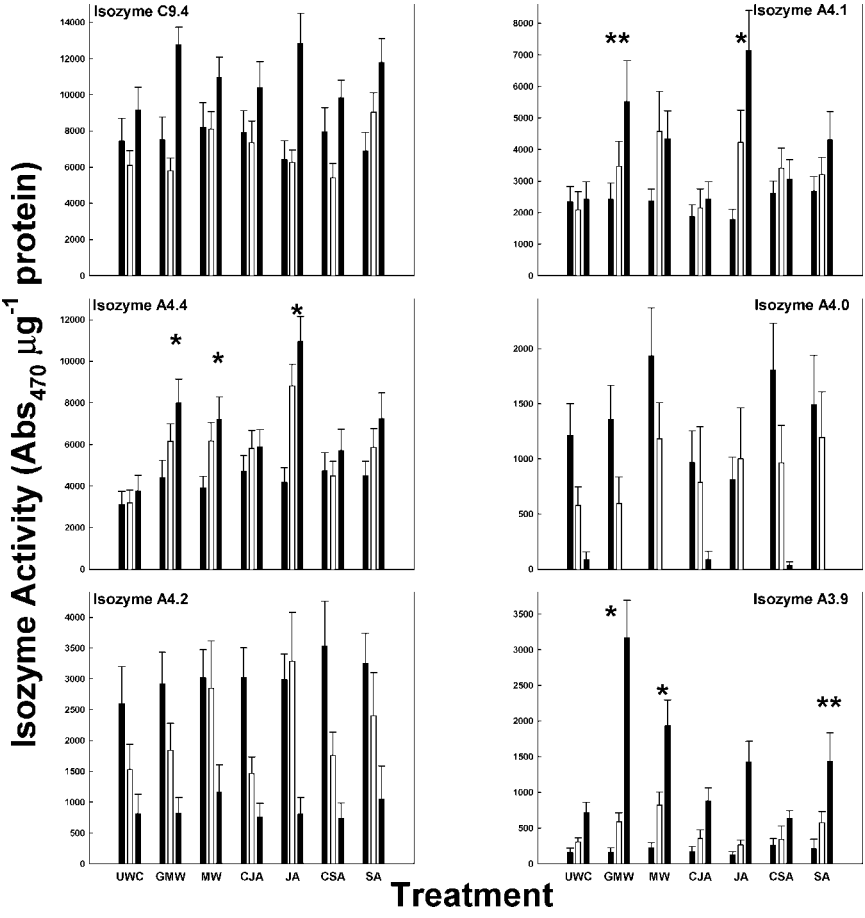

FIG. 3. Means (SE) of the activities of six POD isoforms in greenhouse-grown red oak leaves after 12 hr, 1 wk, and 2 wk of the same treatments indicated in Figure 2. *indicates a significant treatment effect at $P < 0.05$, **indicates $0.05 < P < 0.1$; all isoforms exhibited significant ($P < 0.05$) changes with time.

DISCUSSION

At least 10 POD isozymes were observed in tissue extracts from greenhouse-grown red oak half-siblings and 16 in tissue extracts from field-grown red oaks. These numbers are consistent with a study of POD isozyme variation among and within red oak populations in the eastern US, where 17 total isozymes were noted and 8–12 were found in trees of a given stand (Houston, 1983). This diversity

TABLE 1. POD ISOFORMS FOUND IN FIELD-GROWN RED OAK LEAVES FROM 21 TREES AND THE 6 ISOFORMS QUANTIFIED IN GREENHOUSE STUDY

Field collection isoform identifiction	Isoelectric point	
	Field	Greenhouse
C1	9.87	
C2	9.37	9.4
C3	8.89	
C4	8.21	
A1	5.84	
A2	5.06	
A3	4.82	
A4	4.71	
A5	4.56	
A6	4.37	4.4
A6	4.20	4.2
A7	4.13	4.1
A8	4.06	4.0
A9	3.87	3.9
A10	3.76	
A11	3.68	

of isozymes could provide red oak with flexibility in dealing with common environmental threats such as herbivory, fungal and bacterial pathogen infection, mechanical stress and damage, and air pollutants. In addition, the complex phenolic chemistry of oaks (Li and Hsiao, 1975; Schultz and Baldwin, 1982), may require specific POD isozymes for synthesis or activation (Calderón et al., 1990; Appel, 1993).

In the greenhouse study, activity of red oak POD isozymes varied among half-sib individuals (data not shown) and through time, even within treatments. Variation in isoforms expressed was even greater among field-grown trees. Genetic, biochemical, and developmental factors can all produce this kind of variation within plant species. Loblolly pine cotyledons have as many as 11 isozymes, each of which differs dramatically in activity among seeds of different genotypes (Neuman et al., 1992). Up to 10 isozymes may be found in loblolly needle tissue, but only four of these occur consistently among trees (Snyder and Hamaker, 1978). Flax plants possess four stress-induced and nine constitutive isozymes, some of which arise by differences in posttranslational modification (Fieldes and Gerhardt, 1998). In watermelon seedling stems and cotyledons, novel anionic isozymes appear over the course of development, while others decrease or retain similar levels of activity (Biles and Martyn, 1993). All of these patterns may also be seen in

TABLE 2. DIFFERENCES BETWEEN POD ACTIVITY IN GALL BODIES AND LEAVES FOR 12 WASP AND FLY GALL SPECIES ON RED OAK (*Q. rubra* L.)

Isozyme	# samples with isozyme in leaf or gall[a]	Mean (SE) difference[b]	*T*-statistic	Percent of total isozyme activity[c]
C1	2	−1.24 (1.30)	−1.171	0.2
C2	23	−7.66 (2.58)	−2.961*	26.8
C3	9	−5.92 (5.60)	−1.056	7.1
C4	7	1.23 (0.28)	4.406*	1.2
A1	6	0.40 (0.19)	2.080†	0.3
A2	5	0.49 (0.25)	1.970	0.3
A3	12	0.54 (0.37)	1.476	0.8
A4	8	1.32 (1.38)	0.962	2.9
A5	7	0.58 (0.13)	4.545*	0.3
A6	19	2.62 (0.61)	3.112*	16.8
A7	16	−0.05 (0.44)	−0.092	3.1
A8	22	5.47 (1.55)	3.508*	27.3
A9	16	3.36 (1.46)	2.288*	7.0
A10	22	1.03 (0.52)	1.940†	4.5
A11	6	0.48 (0.17)	2.803*	0.3
A12	15	0.50 (0.14)	3.584*	1.1
Protein content[d]	23	4.24 (1.94)	2.186*	

Note. *T*-statistics were calculated from a paired-difference test for the mean difference: (average host leaf isozyme activity) − (entire gall isozyme activity) for each gall species C = cationic isozyme, A = anionic isozyme.

[a] 23 samples (each sample includes entire gall, galled leaf, and ungalled leaf) were taken from 21 different trees.

[b] Relative absorbance value/unit protein.

[c] The total isozyme activity is a relative measure of the overall prevalence of an isozyme in the entire gall and leaf tissues sampled.

[d] mg protein/g fresh weight.

* $P < 0.05$; † $P < 0.10$.

our northern red oak data. It is likely that we have underestimated the number of significant treatment effects. Many treatments appeared to alter activities of particular isoforms (Figure 3), but individual variation often led to statistical probability values between 0.06 and 0.1.

Activities of all 6 of the red oak isoforms we studied in seedlings appear to be developmentally regulated to some extent (Figure 3). Four increased in activity over time (C9.4, A4.4, A4.1, and A3.9), while the other two decreased (A4.2 and A4.0). Peroxidase isozymes in many other plant species are known to be developmentally regulated (Gaspar et al., 1985), but it is not possible to assign developmental roles to red oak PODs without knowing their specific functions. Some isoforms that increased in activity during our study may have been involved in lignification of the developing leaves (Gaspar et al., 1985; Díaz and Merino, 1998).

Although isozymes A 3.9, A4.4, and A4.1 are all wound-responsive, quantitative differences among them indicate that red oak defensive responses may distinguish among insect wounding, mechanical wounding, and JA treatment. Both GM and mechanical wounding increased the activities of isozymes A4.4 and A3.9, but the response of A3.9 to GM wounding was more pronounced. Isozymes A4.1 and 4.4 responded to both insect wounding and to JA, a signal in the wound response pathway (Zhang and Baldwin, 1997), but these isozymes responded less strongly to mechanical wounding (Figure 3). Isozyme A3.9 failed to change significantly under JA treatment, despite large increases in response to both types of wounding.

Changes in the activities of POD isozymes have been observed in response to pathogen infection and wounding in several plant species (Espelie et al., 1986; Bashan et al., 1987; Lagrimini and Rothstein, 1987; Svalheim and Robertsen, 1990; Ye et al., 1990). In addition, generalized soluble or wall-bound POD activity increases in other plant species in response to wounding (Birecka and Miller, 1974; Kawaoka et al., 1994), pathogens (Bashan et al., 1987), salicylic acid (Rao et al., 1997), and jasmonates (Thaler et al., 1996; Moore et al., 2003). While JA and insect wounding have been found to elevate total soluble POD activity similarly (Choi et al., 1994; Tamari et al., 1995; Zhang and Baldwin, 1997), this study is among the first to suggest that various peroxidase isozymes may be differentially responsive to JA or other signals (Buzi et al., 2004) and the first to find insect-responsive POD isoforms. Our results also indicate that JA does not elicit POD isozymes in exactly the same manner as does gypsy moth herbivory.

Isoform A3.9 activity was enhanced by treatment with SA ($P = 0.055$), a signal more associated with plant defense responses to microbes (Enyedi et al., 1992; Choi et al., 1994; Conti et al., 1996). It is possible, although unlikely, that our trees were incidentally infected by microbes during that particular treatment despite sterilizing the leaf punch, and that A3.9 is both wound- and microbe-responsive. However, no evidence of infection (e.g., necrosis) was observed at any time.

All of the isozymes showing statistically significant increases in response to our greenhouse treatments were anionic. It is possible that cationic isozymes were also induced; additional cationic isozymes are present in red oak and are influenced by galling insects in nature (Allison and Schultz, unpublished), but we were unable to resolve them completely on seedling IEF gels. Although the exact functions of anionic isozymes in red oak are unknown, their induction by wounding is consistent with responses to insects (Felton et al., 1992, Arnason et al., 1994, Dowd, 1994; Dowd et al., 1998a,b). Anionic PODs have been implicated in a variety of leaf-toughening responses, such as lignin biosynthesis (Gaspar et al., 1985; Díaz and Merino, 1998), suberization (Espelie et al., 1986; McDougall, 1993), and cross-linking of cell walls and extensin polymers (Ridge and Osborne, 1970; Everdeen et al., 1988; Bostock and Stermer, 1989). Such toughening processes may decrease the nutritional quality of the plant tissue for herbivores (Bi et al., 1997) as well as

prevent secondary pathogen infection (Vance et al., 1980; Bostock and Stermer, 1989).

Considering the induction in greenhouse seedlings of isozymes A4.4, A4.1, and A3.9, and previous studies of POD wound responsiveness in other plants (e.g., Felton et al., 1994; Bi and Felton, 1995; Bi et al., 1997), we expected total soluble POD activity to increase in response to wounding and JA treatments. Surprisingly, total POD activity was reduced by JA treatment (Figure 1) and was generally unrelated to the isoform values in any clear way. This paradox could be explained by enzyme inhibition in the reaction mixture that is eliminated when isozymes are spatially separated on a gel. More likely, PODs induced by wounding and JA may have greater affinity for *o*-dianisidine than guaiacol, resulting in observable induction only when the former substrate was used on gels. The dip in total POD activity evident after 1 wk in five of seven treatment classes may also be explained by changes in isoforms that were not visualized by our methods; this pattern was not seen in the six measured isoforms (Figure 3).

Activities of various isoforms often differed strongly between the greenhouse seedlings and naturally-occurring trees, and many more isoforms could be identified in field-grown tissues. Cationic isoform C9.4 and anionic A4.1 and A4.2 were unaffected by our seedling treatments, but C9.4 was dramatically suppressed in field-collected galls (compared with matched ungalled leaf tissue), while A4.1 and A4.2 were more active in galls. Suppression and elicitation profiles were characteristic of the insect species involved, although we had to pool results for statistical treatment. Like the differential greenhouse responses to GM, wounding, and JA, this also suggests that red oak POD responses may be unique to the attacking insect. While studies of POD in the context of herbivory have focused on total POD activity (e.g., Cipollini and Redman, 1999; Mayer et al., 2002; Roitto et al., 2003; Traw et al., 2003), the plant pathology literature provides many examples of pathogen-specific POD isoform activity or profiles (Kristensen et al., 1999; Kandan et al., 2002; Tognolli et al., 2002; Maksimov et al., 2003). In some cases, POD isoform function is based on hostplant substrate specificity and the production of specific defenses (Calderón et al., 1990). Transgenic sweetgum (*Liquidambar styraciflua* L.) trees expressing an anionic POD were less palatable to gypsy moth larvae but not all insects (Dowd et al., 1998a). Elevating a single anionic POD in tomato affected some insect herbivores but not others (Dowd et al., 1998b). We suggest that it may be profitable to investigate POD expression profiles in response to specific insects, as well.

We conclude that at least three of the six POD isoforms we observed in greenhouse-grown red oak seedlings respond dynamically and differentially to mechanical wounding, insect wounding, and JA or SA treatments. Furthermore, the activities of these isozymes changed dramatically and differentially over the course of plant development. While plant POD responses to insects are typically measured in terms of total soluble enzyme activity, our results indicate that measurement of

individual isozyme activities may be required to understand fully plant responses to them and other environmental stimuli. Further experiments will be required to determine the exact physiological functions of the POD isozymes present in red oak.

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COMPARATIVE CAPABILITY TO DETOXIFY VEGETABLE ALLELOCHEMICALS BY LARVAL MOSQUITOES

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Abstract—In order to confirm the phytotoxicological basis for the ecological specialization of larval culicine fauna among different subalpine mosquito breeding sites, we compared the capability of six different *Aedes* larval taxa or populations of different ecological origin to detoxify dietary leaf litter originating from the environmental vegetation. Detoxification experiments were performed through *in vitro* digestion of a toxic leaf litter fraction using larval extracts as the enzymatic sources. Comparison of toxicological and detoxifying properties among the different larval samples indicates an association between their tolerance to leaf litter toxicants and their detoxification capability, which vary according to ecological origin. The fact that the detoxifying factor within the larval extracts appears to be a protein-like compound with a molecular weight bigger than 30 kDa suggests the possible involvement of detoxifying enzymes in larval tolerance to leaf litter toxicants. This is congruent with previous biochemical data that suggests the involvement of cytochrome P450 monooxygenase and esterase activities in the detoxification process.

Key Words—Toxic leaf litter, larval *Aedes*, detoxification capability, differential tolerance, ecological specialization.

INTRODUCTION

In plant-insect dietary interactions, plant chemistry is recognized as an important determinant on both an ecological and evolutionary scale. The capability of insects to overcome plant toxic allelochemicals is involved in the ecological discrimination and diversification among those consumers (Berenbaum, 2002). Such a key adaptation is mediated through various detoxifying mechanisms coupled with excretion processes of those allelochemicals, thus allowing radiation and

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colonization of novel ecological niches by tolerant taxa (Johnson, 1999). This metabolic tolerance has been mostly investigated among terrestrial phytophagous taxa (Brattsten, 1992; Snyder and Glendinning, 1996; Berenbaum and Zangerl, 1999), and is far less known in the aquatic medium (Sota, 1993). In this medium, plant detritus acts as a main food source for numerous insect taxa, including larval mosquitoes (Clements, 1992; Walker et al., 1997), through an indirect plant-insect interaction called detritivory.

The biological impact of detritivory is particularly important in subalpine mosquito breeding sites because of the differential toxicity exerted against culicine larval fauna by the decomposed leaf litter coming from arborescent vegetation (Rey et al., 1996). This food source may exert a deleterious effect against the midgut epithelium of sensitive consumers (David et al., 2000c), due to toxic water-insoluble lignin–polypeptidic complexes formed during a 10-mo complex decaying process (David et al., 2000b, 2001; Tilquin et al., 2002b). Comparative ecotoxicological investigations performed together with biochemical studies have suggested that the larval tolerance to crude toxic leaf litter may involve cytochrome P450 monooxygenase (P450) and esterase activities (Meyran et al., 2002), which are often implicated in insect tolerance to allelochemicals (Brattsten, 1988; Amichot et al., 1998; Scott et al., 1998). However, no assocation between the levels of detoxifying activities and the rates of toxic leaf litter degradation has been established, because of the lack of isolated toxic molecules available for direct *in vitro* assays. Hot water extraction of the toxic compounds from crude leaf litter allowed us not only to concentrate the toxicity into an insoluble toxic fraction (IF) (Tilquin et al., 2002a), but also to provide the IF as a possible substrate for experimental assays.

In this paper, the possible relation between differential larval tolerance to IF extracted from crude toxic leaf litter and the capability to detoxify IF *in vitro* was investigated in several mosquito taxa and populations of various origin (ecotypes). Such an association might allow us to confirm the phytotoxicological basis for ecological specialization of subalpine larval culicine fauna among breeding sites surrounded by a chemically different vegetation.

METHODS AND MATERIALS

Plant and Insect Material

Crude toxic 10-mo decomposed poplar leaf litter samples were collected within the old leaf litter layer (Green et al., 1993) of a subalpine floodplain forest that included numerous mosquito breeding sites, as described by Pautou and Girel (2000). Freshly collected samples were lyophilized, reduced into 0.5 mm mesh homogeneous powder, and stored at -80°C until use. A toxic IF was extracted from each standard crude sample (70 mg) according to the procedure of Tilquin et al. (2002a). After hot water treatment (60°C for 1 hr), the resulting aqueous

extract was filtered, allowed to stand for 24 hr to gradually precipitate the IF, and then was recovered by centrifugation (5 min at 5,000*g*). The IF sample was immediately used for detoxification experiments.

In order to evaluate the efficacy of the detoxification procedure, IF toxicity was determined in bioassays before and after detoxification experiments. Bioassays were conducted on fourth instar *Aedes aegypti* larvae (Bora–Bora strain) raised in the laboratory (Rey et al., 1999). This laboratory source was preferred to field taxa because of its standardized tolerance to crude, toxic, decomposed leaf litter (David et al., 2000c) and its constant availability.

The capability to detoxify IF was comparatively checked among fourth instar larvae of *A. aegypti* and several subalpine *Aedes* taxa or ecotypes originating from various environments and known to have different dietary tolerances to crude toxic leaf litter (Rey et al., 2000b). For *A. cantans* Meigen, one ecotype was chosen, which originated from a polyphenol-poor herbaceous habitat and was poorly tolerant to crude toxic litter. For *A. cataphylla* Dyar and *Aerusticus* Rossi, two ecotypes were chosen: ecotype 1, originating from a polyphenol-rich woody habitat and highly tolerant to crude toxic leaf litter; ecotype 2, originating from an polyphenol-poor herbaceous habitat, and poorly tolerant to crude toxic leaf litter.

Bioassays. In all cases, larval tolerance to IF was checked by using the standard bioassay procedure of Tilquin et al. (2002a). Bioassay were performed in triplicate, at 25°C, on samples including 20 identically-sized and aged larvae exposed until 100% mortality to a standard IF sample suspended in 10 ml of water adjusted to pH 7.5. Controls were reared in tap water. Larval mortality was checked every hour during the bioassay procedure in order to constract a mortality curve. Larval tolerance was expressed as the time necessary to reach 50% mortality (LT_{50}) obtained after curve fitting (Ffrench-Constant and Roush, 1990).

Detoxification Experiments. The detoxification capability of different *Aedes* larval samples was comparatively tested through *in vitro* digestion of IF by larval extracts. Identically-sized and aged freshly collected larvae were frozen at −80°C. They were thawed and centrifuged for 10 min at 5,000*g* to recover the supernatant. The larval extracts thus collected were frozen at −80°C until use.

In order to compare the detoxification capability among the different samples, a standard efficient dose of larval extract was chosen (see Results). This dose, corresponding to the minimum amount of larval extract necessary to cause *in vitro* detoxification, was adjusted using the most tolerant species, i.e., *Ae. rusticus* ecotype 1.

Each in *vitro* IF assay was carried out in triplicate, for 6 hr at 35°C, in Eppendorf tubes shaken every 2 hr and containing a standard toxic IF sample resuspended into 150 μl of aqueous larval extract (corresponding to around 200 mg of fresh material) adjusted to pH 6 with 150 μl of MES/TRIS buffer (300 mM). The reaction was stopped by removing the supernatant by centrifugation (5,000*g* for 5 min), then the treated IF was rinsed twice in 1-ml distilled

water. After centrifugation, the IF pellet was resuspended in 10-ml of water before bioassays.

The detoxification capability of other *Aedes* larval samples was comparatively tested according to the protocol described for *A. rusticus*, using 150 μl of larval extract per assay. The protein contents of all tested samples were determined by using the Bradford method, and ranged from 4.5 to 6.5 mg of protein per assay (data not shown). Detoxification results were expressed as the residual IF toxicity after treatment. In all cases, controls were carried out on standard toxic IF samples submitted to the same treatment without enzymatic extract.

Evidence for a Possible Protein Feature of the Detoxifying Factor in Larval Extracts

Denaturing Experiment. Denaturing experiments were performed on 500 μl of *A. rusticus* (ecotype 1) larval extracts treated at 100°C for 5 min. Each denatured extract was then centrifuged (5,000*g* for 5 min), and the supernatant was collected then used for IF detoxification assay as described above.

Fractionation on a Filtration Gel. The size of the molecules involved in the detoxifying factor was investigated through Sephadex fractionation. 2.5 Ml of *A. rusticus* (ecotype 1) larval extract were pored onto a Sephadex column (G50, fine Pharmacia, fractionation range 5,000–30,000) buffered to pH 6 with 150 mM MES/TRIS and calibrated with Dextran blue (MW: 2,000,000) and cyanidin-3-glucoside (MW: 449). The two eluted fractions (i.e., one corresponding to molecular weights larger than 30 kDa, the other corresponding to molecular weights smaller than 30 kDa) were immediately freeze-dried. After solubilization in 2.5 ml of distilled water, 150 μl of each fraction were used for the IF detoxification assay, as described above.

RESULTS

Relation Between Tolerance to the Leaf Litter Insoluble Fraction and Detoxification Capability Among Aedes Larvae

The larval tolerance to the toxic IF isolated from decomposed leaf litter varied according to the taxon and/or ecotype (Table 1). Among the different taxa, *A. aegypti* was the most sensitive. Among the different ecotypes, ecotypes 1, coming from habitats with a polyphenol-rich woody vegetation, were more tolerant than ecotypes 2, coming from habitats with a polyphenol-poor herbaceous vegetation.

The larval extract of *A. rusticus* (ecotype 1) appeared efficient in detoxifying the IF, with a dose-response effect (Figure 1). IF toxicity was unaffected in the controls; however, nearly complete detoxification was obtained with 300 μl of extract. A 5% larval mortality was observed with 150 μl of larval extract, so this

TABLE 1. DIFFERENTIAL TOLERANCE OF *Aedes* TAXA (OR ECOTYPES) TO IF

Taxa (ecotypes)	LT_{50} (hr)	Polyphenolic richness of the environmental vegetation
Ae. aegypti	1	(laboratory species)
Ae. cataphylla (ecotype 2)	3	−
Ae. rusticus (ecotype 2)	5	−
Ae. cantans	13	+
Ae. cataphylla (ecotype 1)	16.5	+
Ae. rusticus (ecotype 1)	24	+

Note. Expressed as the time necessary to reach 50% mortality in the bioassays (LT_{50} values obtained by curve fitting), in relation with the polyphenol richness of the environmental vegetation, as determined by Rey et al. (2000); polyphenol-poor sites (average polyphenolic content 3 mg equivalent gallic acid/g of leaves) are noted −; polyphenol-rich sites (average polyphenolic content 13 mg equivalent gallic acid/g of leaves) are noted +.

dose was chosen as the standard dose to compare the detoxification capability of the different *Aedes* larval extracts.

A strong association between larval tolerance to the IF and detoxification capability was established from the comparison between toxicological and detoxifying performances among the six *Aedes* samples (Figure 2). There was a gradual increase in larval detoxification capability from the less tolerant (i.e., *A. aegypti*) to the most tolerant (i.e., *A. rusticus*, ecotype 1).

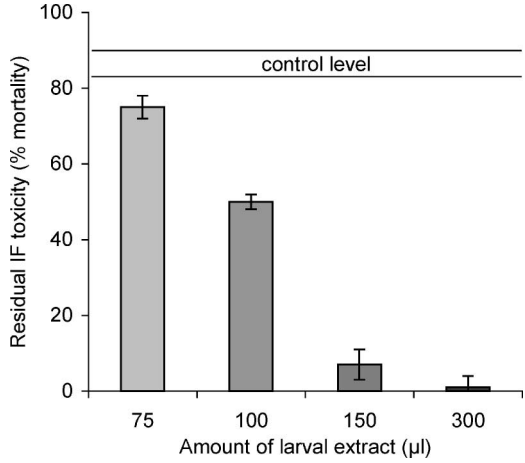

FIG. 1. Detoxifying performances of different amounts of *Aedes rusticus* larval extract, expressed as the residual IF toxicity (% mortality ± SE calculated from triplicate) measured after treatment. The control level corresponds to the mortality observed with untreated toxic IF.

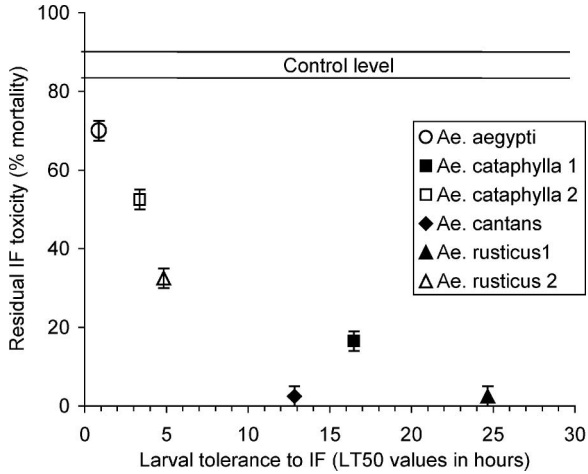

FIG. 2. Detoxification capability of the different *Aedes* taxa or ecotypes (1, 2) in relation with their tolerance to IF. Larval tolerance is expressed as the time necessary to reach 50% mortality (LT_{50}). The detoxification capability corresponds to the residual IF toxicity measured after treatment with 150 μl of larval extract. The control level corresponds to the mortality observed with toxic IF.

Evidence for a Possible Protein Feature of the Detoxifying Factor in Larval Extracts. After heat-denaturation, *A. rusticus* larval extracts were totally ineffective in detoxifying the IF, compared to the native extract (Table 2). This suggested the involvement of a protein-like factor in the detoxification process. Moreover, the fractionation results (Table 3) showed no detoxifying effect in the fraction with low molecular weight molecules (<30 kDa), whereas the fraction with high molecular weight fraction (>30 kDa) appeared as efficient in alleviating the IF toxicity as the whole larval extract.

TABLE 2. COMPARATIVE DETOXIFICATION CAPABILITY OF *A. Rusticus* LARVAL EXTRACTS BEFORE (NATIVE EXTRACT) AND AFTER HEAT-DENATURATION (DENATURED EXTRACT)

Residual IF toxicity (% mortality)		
Control	Native extract	Denatured extract
85 ± 6	10 ± 5	82 ± 2

Note. Expressed as the residual IF toxicity (% mortality ± SE calculated from triplicate).

TABLE 3. COMPARATIVE DETOXIFICATION CAPABILITY OF *A. rusticus* LARVAL EXTRACTS BEFORE (WHOLE EXTRACT) AND AFTER FRACTIONATION ON A SEPHADEX COLUMN

Residual toxicity (% mortality)			
Control	Whole extract	MW < 30 kda	MW > 30 kda
86 ± 3	6 ± 2	78 ± 4	3 ± 2

Note. Expressed as the residual IF toxicity (% mortality ± SE calculated from triplicate).

DISCUSSION

The current comparison allowed us to associate the differential tolerance of *Aedes* larvae to leaf litter with their differential capability to detoxify this food source. These toxicological performances are also related to larval ecological origin. Characterization within larval extracts of a heat-sensitive detoxifying factor with a molecular weight above to 30 kDa is congruent with the possible involvement of detoxifying enzymes in larval dietary tolerance to leaf litter toxicants.

Because of the structural complexity of the toxic IF (Tilquin et al., 2002b), our investigations are not sufficient to provide characterized leaf litter toxic molecules suitable for direct *in vivo* or *in vitro* experiments, as have been reported in only few plants (Ma et al., 1994; Johnson, 1999; Ruuhola et al., 2001). However, the possibility of IF extraction from leaf litter gave us the opportunity to provide a substrate suitable for *in vitro* detoxification by larval extracts used as the enzyme sources.

The results obtained from those detoxification experiments emphasize the differential detoxifying ability of larval *Aedes* taxa, which is congruent with previous bioassay results using crude toxic leaf litter (Tilquin et al., 2002b; David et al., 2002b). The association between the IF bioassay and detoxification indicates the importance of metabolic processes, rather than behavioral mechanisms, in larval tolerance to dietary leaf litter toxicants, as has been suggested by David et al. (2002a).

Moreover, as such performances vary according to larval habitat, the differential capability to metabolize leaf litter toxicants appears to be reasonable explanation for patterns of ecological differentiation among larval mosquito taxa or ecotypes throughout subalpine breeding sites. Such an association between larval toxicological performances and chemical characteristics of the environmental vegetation is congruent with our previous ecotoxicological comparison (Rey et al., 2000b). Thus, the culicine ecological differentiation among subalpine breeding sites appears to result from their differential detoxifying capabilities (David et al., 2000a; Tilquin et al., 2002b). The impact of such larval toxicological traits determines the distribution of adult populations (Clements, 1992).

Possible Involvement of Detoxifying Enzymes in Larval Tolerance to Leaf Litter Toxicants. As the association between lignin-like and peptidic compounds in the toxic fraction is the basis of dietary leaf litter toxicity against larval mosquitoes (Tilquin et al., 2002b), dissociation of such complexes may be the result of detoxification processes. An *in vitro* enzymatic detoxification of the IF has been experimentally obtained only through action of laccase (Tilquin et al., 2002a), which is a well-known lignin degrading agent (Reid, 1995). Comparable dissociation of those toxic lignin–peptidic complexes in larval midguts may necessitate enzymatic processes quantitatively and qualitatively different in tolerant and sensitive larvae. A differential detoxifying enzymatic process is suggested by the association observed between bioassay performances and the activity levels of detoxifying enzymes, such as P450s and esterases, measured within larval extracts used as enzyme sources (Meyran et al., 2002). However, those detoxifying activities were measured *in vitro* by using universal substrates (i.e., ethoxycoumarin-*O*-deethylase and testosterone for P450, and α- and β-naphthyl acetate for esterases), which are structurally different from the leaf litter allelochemicals.

The involvement of P450s and esterases in our larval extracts capable of detoxifying *in vitro* the IF is suggested by several arguments. The protein-like feature and molecular weight interval of the detoxifying factor appear to be related to P450s (i.e., ranging from 45 to 57 kDa; Keserü, 1998) and esterases (i.e., ranging from 50 to 70 kDa; Heyman, 1980). Also, those detoxifying enzymes, located mainly in the midgut and fat body, are generally induced through successive detoxification phases, in order to overcome toxicity (Francis et al., 2001). Such allelochemicals may qualitatively and quantitatively modulate the detoxifying enzyme response (Snyder, 1998). An adaptative plasticity appears to be evident from our comparison of the detoxifying efficacy between larval extracts originating from different ecotypes within a given larval culicine taxon (e.g., *A. cataphylla*, *A. rusticus*). This is congruent with the differential P450 and esterase activity levels previously observed among the same ecotypes (David et al., 2000a). However, the clear involvement of P450s and esterases in the detoxification of leaf litter toxicants by larval *Aedes* remains difficult to establish, because of the structural and functional diversity of those enzyme families, which may be involved in the detoxification of various other xenobiotics and also in processes other than xenobiotic metabolism (Lindroth, 1991; Feyereisen, 1999).

The involvement of such enzymes in the dissociation of leaf litter toxic lignin–peptidic complexes is not surprising, as numerous P450 and esterase activities are frequently involved in the detoxification of plant dietary allelochemicals (Lindroth, 1991; Feyereisen, 1999). Moreover, P450s are known for their versatility and broad substrate specificity (Mansuy, 1998), and there is an abundance of ester groups in the polyphenolic part of the leaf litter toxicants (David et al., 2001). However, other detoxifying enzymes, such as glutathione S-transferases, known to have molecular weights between 50 and 60 kDa (Fournier et al., 1992), may also have a role. Such

an involvement was observed in the detritivorous planktonic crustacean fauna found in those mosquito breeding sites (Rey et al., 2000a), and, more generally, in numerous plant–insect dietary interactions (Yu, 1999; Vanhaelen et al., 2001).

This study shows the significance of environmental phytochemicals as habitat components involved in ecological discrimination among subalpine mosquito taxa or ecotypes. Beside natural toxic food sources, such as arborescent leaf litter, several other xenobiotics, such as pollutants, may interfere with habitat preference of culicine larval fauna (Suwanchaichinda and Brattsten, 2002). This is currently being investigated in our laboratory.

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STRUCTURAL DIVERSITY AND DEFENSIVE PROPERTIES OF NORDITERPENOID ALKALOIDS

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Abstract—We have tested the insect antifeedant and toxic activity of 43 norditerpenoid alkaloids on *Spodoptera littoralis* and *Leptinotarsa decemlineata* including eserine (physostigmine), anabasine, and atropine. Antifeedant effects of the test compounds were structure- and species-dependent. The most active antifeedants to *L. decemlineata* were 1,14-diacetylcardiopetaline (**9**) and 18-hydroxy-14-*O*-methylgadesine (**33**), followed by 8-*O*-methylconsolarine (**12**), 14-*O*-acetyldelectinine (**27**), karakoline (**7**), cardiopetaline (**8**), 18-*O*-demethylpubescenine (**13**), 14-*O*-acetyldeltatsine (**18**), takaosamine (**21**), ajadine (**24**), and 8-*O*-methylcolumbianine (**6**) (EC_{50} <1 $\mu g/cm^2$). This insect showed a moderate response to atropine. *S. littoralis* had the strongest antifeedant response to **24**, **18**, 14-*O*-acetyldelcosine (**19**), and delphatine (29) (EC_{50} <3 $\mu g/cm^2$). None of the model substances affected the feeding behavior of this insect. The most toxic compound to *L. decemlineata* was aconitine (**1**), followed by cardiopetalidine (**10**) (% mortality >60), 14-deacetylpubescenine (**14**), 18-*O*-benzoyl-18-*O*-demethyl-14-*O*-deacetylpubescenine (**17**), 14-*O*-acetyldelcosine (**19**), 14-deacetylajadine (**25**) and methyllycaconitine (**30**)

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(% mortality >45). Orally injected *S. littoralis* larvae were negatively affected by **1**, cardiopetaline (**8**), **10**, 1,14-*O*-acetylcardiopetalidina (**11**), **12**, **14**, 1,18-*O*-diacetyl-19-oxo-gigactonine (**41**), olivimine (**43**), and eserine in varying degrees. Their antifeedant or insecticidal potencies did not parallel their reported nAChR binding activity, but did correlate with the agonist/antagonist insecticidal/antifeedant model proposed for nicotininc insecticides. A few compounds [**14**, tuguaconitine (**38**), 14-demethyldelboxine (**40**), **19**, dehydrodelsoline (**36**), 18-*O*-demethylpubescenine (**13**), **41**, **9**, and delcosine (**23**)] had selective cytotoxic effects to ward insect-derived Sf9 cells. None were cytotoxic to mammalian CHO cells and none increased *Trypanosoma cruzi* mortality. The selective cytotoxic effects of some structures indicate that they can act on biological targets other than neuroreceptors.

Key Words—*Aconitum*, *Consolida*, *Delphinium*, norditerpene alkaloids, insecticidal, cytotoxic structure–activity relationships.

INTRODUCTION

Species of the plant genera *Aconitum* and *Delphinium* are known sources of diterpenoid alkaloids of pharmacological and economic importance due to cattle poisoning (Atta-ur-Rahman and Choudary, 1995; see Panter et al., 2002). These alkaloids act as potent nicotinic cholinergic receptor (nAcChR) agonists and antagonists in invertebrates, vertebrates, and insects (see Seitz and Ameri, 1998; Panter et al., 2002).

The insecticidal and antifeedant activity of some diterpene alkaloids (Jennings et al., 1986; González-Coloma et al., 1998; Ulubelen et al., 2001), and the presence of the highest toxic alkaloid levels in the leaves and stems of *Delphinium barbeyi* early in the growing season (Ralphs et al., 2002; Ralphs and Gardner, 2003) suggest a defensive role in young plant tissues, but only a few structures have been investigated for their antifeedant effects.

We have investigated the defensive properties (insect antifeedant and toxic effects) of 43 norditerpene alkaloids from several chemical classes against the polyphagous *Spodoptera littoralis* (Boisduval) and the Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say), to establish structure–activity relationships (SAR). We have also tested the toxicity of these compounds on two biological models lacking neurotransmission (insect Sf9 and mammalian CHO cells and *Trypanosoma cruzi* epimastigotes).

To establish a comparative model, we included eserine (physostigmine), anabasine, and atropine in this study. Eserine is an inhibitor of acetylcholinesterase and also inhibits insect and vertebrate nAcChRs with insecticidal effects (see Liu et al., 1995). This compound interferes with the neurotoxic action of norditerpene alkaloids, reducing toxicity and preventing death from larkspur poisoning in cattle (Stegelmeier et al., 1998). Anabasine (neonicotine) is a selective nAChR agonist for insects vs. vertebrates with insecticidal activity (Sultana et al., 2002). Atropine

is a competitive muscarinic antagonist at central and peripheral synapses in vertebrates (Knuepfer and Gan, 1999) that also interferes with norditerpene alkaloid toxicity in sheep (Panter et al., 2002).

METHODS AND MATERIALS

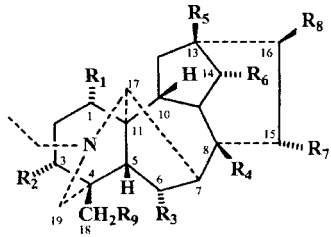
General. Optical rotations and IR spectra were determined at room temperature using a Perkin-Elmer 241 polarimeter and Perkin Elmer 1600 FT spectrometer respectively. NMR spectra were measured on a Bruker AMX2 500 MHz spectrometer with pulsed field gradient, using the solvent as an internal standard ($CDCl_3$, at δ_H 7.26 and δ_C 77.0). Exact mass measurements and EIMS were recorded on an Autospect instrument at 70 eV. Silica gel (15111, 7741, 5554, and 5715) and alumina (1077 and 5581) from Merck were used for column chromatography. Alkaloids were visualized on TLC with Dragendorff's reagent. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was from Sigma-Aldrich. Parasite and cell viability were measured with a microplate reader (SLT Lab Instruments, Austria).

Plant Material. Plant collection and identification are described in (González et al., 1981, 1986; De la Fuente and Reina, 1990; De la Fuente and Ruiz-Mesia, 1994; Grandez et al., 2002; Alba et al., in press a,b)

Compounds. Alkaloids (Figures 1–4) were isolated from *Aconitum napellus* L. subsp. *Castellanum* (**1**, **2**, **3**, and **5**), *A. compactum* Reicheng (**3** and **5**), *A. nevadense* Vechtr. (**3**, **4**, and **5**), *A. vulparia* subsp. *Neapolitanum* (**36**), *Delphinium verdunense* Balb. (syn *D. cardiopetalum* DC.) (**7**, **8**, **10**, and **32**), *D. pentagynum* Lam. (**7**, **31**), *D. montanum* DC. (**3**, **5**, **7**, **22**, **23**, **30**, and **32**), *Consolida oliveriana* Schöd. (**6**, **16**, **29**, **42**, and **43**), *C. pubescens* DC. (**15**, **19**, and **28**), *C. aconiti* Lindlcy (**26**), and several chemotypes of *C. orientalis* Gay subsp. *orientalis* (Guadalajara, Spain: **12**, **14**, **17**, **24**, **25**, **27**, **37**, **39**, and **40**; Cuenca, Spain: **20**, **22**, **23**, **33**, and **35**; Teruel, Spain: **34** and **38**, and Bolu, Turkey: **13**, **18**, and **21**). Spectroscopic data and references are in the Supplementary data section. The semisynthetic compounds **9**, **11**, and **41** were prepared from **8**, **10**, and **22**, respectively. 1,18-*O*-Diacetylgigactonine was prepared from **22** as a previous step to the preparation of **41**.

*1,14-O-Diacetylcardiopetaline (**9**) 1,14-O-Diacetylcardiopetalidine (**11**), and 1,18-O-Diacetylgigactonine*

Cardiopetaline (**8**, 14.4 mg), cardiopetalidine (**10**, 12.2 mg), and gigactonine (**22**, 13.6 mg) were treated with a mixture of dry pyridine (0.4 ml) and Ac_2O (0.4 ml) for 24 hr at room temperature. The reaction mixture was chromatographed over



Aconitine (**1**) ; $R_1 = R_3 = R_8 = R_9 = OMe$; $R_2 = R_5 = R_7 = OH$; $R_4 = OAc$; $R_6 = OBz$

3-Acetylaconitine (**2**) ; $R_1 = R_3 = R_8 = R_9 = OMe$; $R_2 = R_4 = OAc$; $R_5 = R_7 = OH$; $R_6 = OBz$

8-*O*-Ethyl-14-Benzoylaconine (**3**); $R_1 = R_3 = R_8 = R_9 = OMe$; $R_2 = R_5 = R_7 = OH$; $R_4 = OCH_2CH_3$; $R_6 = OBz$

8-*O*-Ethylaconine (**4**); $R_1 = R_3 = R_8 = R_9 = OMe$; $R_2 = R_5 = R_6 = R_7 = OH$; $R_4 = OCH_2CH_3$

Neoline (**5**) ; $R_1 = R_4 = R_6 = OH$; $R_2 = R_5 = R_7 = H$; $R_3 = R_8 = R_9 = OMe$

8-*O*-Methylcolumbianine (**6**); $R_1 = R_6 = R_9 = OH$; $R_2 = R_3 = R_5 = R_7 = H$; $R_4 = R_8 = OMe$

Karakoline (**7**) ; $R_1 = R_4 = R_6 = OH$; $R_2 = R_3 = R_5 = R_7 = R_9 = H$; $R_8 = OMe$

Cardiopetaline (**8**) ; $R_1 = R_4 = R_6 = OH$; $R_2 = R_3 = R_5 = R_7 = R_8 = R_9 = H$

1,14-Diacetylcardiopetaline (**9**); $R_1 = R_6 = OAc$; $R_4 = OH$; $R_2 = R_3 = R_5 = R_7 = R_8 = R_9 = H$

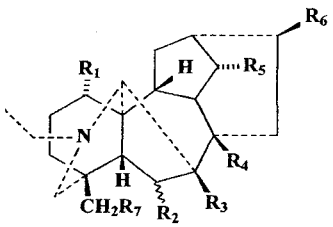
FIG. 1. Aconitne-type structures.

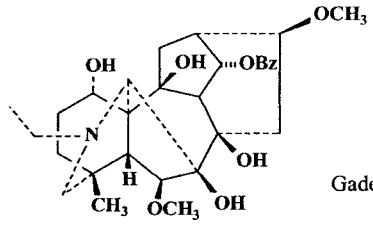
neutral alumina and eluted with EtOAc to afford **9** (17.1 mg, 97.2%), **11** (7.7 mg, 52%), and 1,18-*O*-diacetylgigactonine (10.8 mg, 79.4%).

Compound **9**. ^{1}H NMR ($CDCl_3$, 500 MHz, δ): 0.75 (3H, s, H-18), 1.07 (3H, t, $J = 7.2$ Hz, H-21), 2.00 (3H, s, $OCOCH_3$), 2.07 (3H s, $OCOCH_3$), 3.41 (H, br s, H-17), 4.75 (H, t, $J = 5.1$ Hz, H-14β), 4.86 (H, dd, $J = 10.8, 6.9$ Hz, H-1β).

Compound **11**. ^{1}H NMR ($CDCl_3$, 500 MHz, δ): 0.95 (3H, s, H-18), 1.13 (3H, t, $J = 7.0$ Hz, H-21), 2.11 (3H, s, $OCOCH_3$), 2.71 and 3.05 (H, m, H-20A and H-20B), 3.71 (H, d, $J = 5.3$ Hz, H-1β), 3.90 (H, s, H-17), 4.77 (H, t, $J = 5.0$ Hz, H-14β).

1,18-O-Diacetylgigactonine. ^{1}H NMR ($CDCl_3$, 500 MHz, δ): 1.09 (3H, t, $J = 7.1$ Hz, H-21), 1.70 (1H, dd, $J = 15.5, 6.5$ Hz, H-15β), 1.75 (1H, dd, $J = 11.1, 4.3$ Hz, H-12α), 1.76 (1H, d, $J = 2.1$ Hz, H-5), 2.05, 2.07 (3H each, s, 2 $\times$ $OCOCH_3$), 2.33 (1H, dd, $J = 8.3, 4.3$ Hz, H-13), 2.47 (1H, dd, $J = 11.7, 1.9$ Hz, H-19β), 2.61 (1H, dd, $J = 15.5, 9.0$ Hz, H-15α), 2.66 (1H, d, $J = 11.8$ Hz, H-19α), 2.76 (1H, dq, $J = 14.0, 7.0$ Hz, H-20B), 2.98 (1H, d, $J = 2.4$ Hz, H-17), 3.00 (1H, dq, $J = 14.0, 7.3$ Hz, H-20A), 3.07 (1H, dd, $J = 6.7, 4.9$ Hz, H-9), 3.14 (1H, dd, $J = 8.6, 6.8$ Hz, H-16α), 3.34 (3H, s, H-16′),





Gadenine (**31**)

Cardiopetalidine (**10**); $R_1 = R_3 = R_4 = R_5 = OH$; $R_2 = R_6 = R_7 = H$

1,14-*O*-Acetylcardiopetalidina (**11**); $R_1 = R_5 = OAc$; $R_3 = R_4 = OH$; $R_2 = R_6 = R_7 = H$

8-*O*-Methylconsolarine (**12**); $R_1 = R_3 = R_5 = OH$; $R_2 = \alpha OH$; $R_4 = R_6 = OMe$; $R_7 = H$

18-*O*-Demethylpubescenine (**13**); $R_1 = R_3 = R_7 = OH$; $R_2 = \alpha OH$; $R_4 = R_6 = OMe$; $R_5 = OAc$

14-Deacetylpubescenine (**14**); $R_1 = R_3 = R_5 = OH$; $R_2 = \alpha OH$; $R_4 = R_6 = R_7 = OMe$

Pubescenine (**15**); $R_1 = R_3 = OH$; $R_2 = \alpha OH$; $R_4 = R_6 = R_7 = OMe$; $R_5 = OAc$

Consolidine (**16**); $R_1 = R_3 = OH$; $R_2 = \alpha OH$; $R_4 = R_5 = R_6 = R_7 = OMe$

18-*O*-Benzoyl-18-*O*-Demethyl-14-*O*-Deacetylpubescenine (**17**); $R_1 = R_3 = R_5 = OH$; $R_2 = \alpha OH$; $R_4 = R_6 = OMe$; $R_7 = OBz$

14-*O*-Acetyldeltatsine (**18**); $R_1 = R_3 = OH$; $R_2 = \beta OMe$; $R_5 = OAc$; $R_4 = R_6 = R_7 = OMe$

14-*O*-Acetyldelcosine (**19**); $R_1 = R_3 = R_4 = OH$; $R_2 = \beta OMe$; $R_5 = OAc$; $R_6 = R_7 = OMe$

Delsoline (**20**); $R_1 = R_3 = R_4 = OH$; $R_2 = \beta OMe$; $R_5 = R_6 = R_7 = OMe$

Takaosamine (**21**); $R_1 = R_3 = R_4 = R_5 = R_7 = OH$; $R_2 = \beta OMe$; $R_6 = OMe$

Gigactonine (**22**); $R_1 = R_3 = R_4 = R_7 = OH$; $R_2 = \beta OMe$; $R_5 = R_6 = OMe$

Delcosine (**23**); $R_1 = R_3 = R_4 = R_5 = OH$; $R_2 = \beta OMe$; $R_6 = R_7 = OMe$

Ajadine (**24**); $R_1 = R_6 = OMe$; $R_2 = \beta OMe$; $R_3 = R_4 = OH$; $R_5 = OAc$; $R_7 = OCOPhNHAc$

14-Deacetylajadine (**25**); $R_1 = R_6 = OMe$; $R_2 = \beta OMe$; $R_3 = R_4 = R_5 = OH$; $R_7 = OCOPhNHAc$

Lycoctonine (**26**); $R_1 = R_5 = R_6 = OMe$; $R_2 = \beta OMe$; $R_3 = R_4 = R_7 = OH$

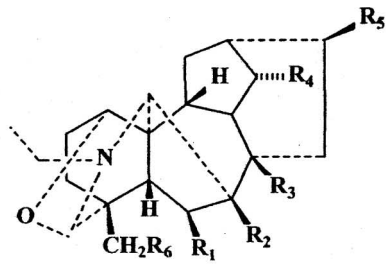
14-*O*-Acetyldelectinine (**27**); $R_1 = R_6 = OMe$; $R_2 = \beta OMe$; $R_3 = R_4 = R_7 = OH$; $R_5 = OAc$

Browniine (**28**); $R_1 = R_6 = R_7 = OMe$; $R_2 = \beta OMe$; $R_3 = R_4 = R_5 = OH$

Delphatine (**29**); $R_1 = R_5 = R_6 = R_7$ OMe; OMe; $R_2 = \beta OMe$; $R_3 = R_4 = OH$

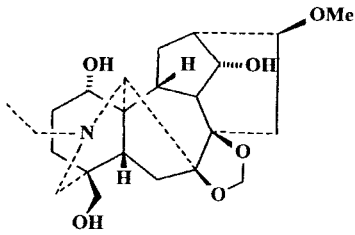
Methyllicaconitine (**30**); $R_1 = R_5 = R_6 = OMe$; $R_2 = \beta OMe$; $R_3 = R_4 = OH$; $R_7 =$ 

FIG. 2. Lycoctonine-type structures.

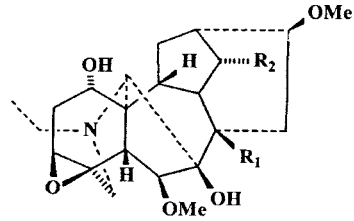


14-*O*-Benzoylgadesine (**32**); $R_1 = R_5 = OMe$; $R_2 = R_3 = OH$; $R_4 = OBz$; $R_6 = H$
18-Hydroxy-14-*O*-Methylgadesine (**33**); $R_1 = R_4 = R_5 = OMe$; $R_2 = R_3 = R_6 = OH$
Dehydrotakaosamine (**34**); $R_1 = R_5 = OMe$; $R_2 = R_3 = R_4 = R_6 = OH$
18-*O*-Methoxygadesine (**35**); $R_1 = R_5 = R_6 = OMe$; $R_2 = R_3 = R_4 = OH$
Dehydrodelsoline (**36**); $R_1 = R_4 = R_5 = R_6 = OMe$; $R_2 = R_3 = OH$

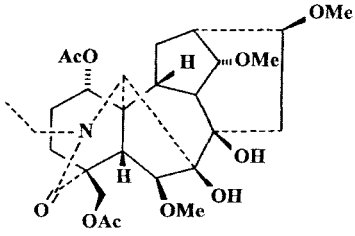
FIG. 3. Gadesine-type structures.



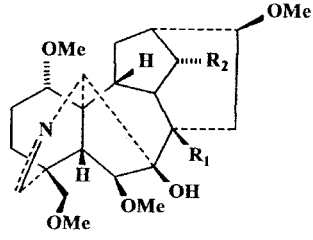
Ajadelphinine (**37**)



Tuguaconitine (**38**); $R_1 = OH$, $R_2 = OMe$
14-Demethyltuguaconitine (**39**); $R_1 = R_2 = OH$
14-Demethyldelboxine (**40**); $R_1 = OMe$; $R_2 = OH$



1,18-*O*-Diacetyl-19-oxo-gigactonine (**41**)



Olividine (**42**); $R_1 = OMe$; $R_2 = OAc$
Olivimine (**43**); $R_1 = OH$; $R_2 = OMe$

FIG. 4. Miscellaneous structures.

3.38 (3H, s, H-6′), 3.40 (3H, s, H-14′), 3.57 (1H, t, $J = 4.6$ Hz, H-16α), 3.89 (1H, s, H-6α), 3.91 (1H, d, $J = 11.2$ Hz, H-18B), 3.98 (1H, d, $J = 11.3$ Hz, H-18A), 4.00 (1H, s, disappeared with D_2O, OH), 4.75 (1H, dd, $J = 10.2$, 7.3 Hz, H-1β).

*1,18-O-Diacetyl-19-oxo-gigactonine (***41***).* An aliquot of 6.6 mg of I_2 (from a 165.5 mg of I_2 in 5 ml of benzene solution) was gradually added (4 hr) to a mixture of 1,18-*O*-diacetylgigactonine (16.7 mg), dissolved in benzene (1.0 ml) and sodium bicarbonate (35.0 mg). Excess reagent was removed with sodium bisulphite. The dry residue was purified on a neutral alumina column eluted with $CHCl_3$. Further purification by PTLC (20 cm × 20 cm × 0.2 mm, neutral alumina plate) eluted with a mixture of EtOAc:MeOH (19:1) gave compound **41** (2.4 mg, 15%). IR ($CHCl_3$) νmax 3445, 1737, and 1236 (ester group), 1637 and 1122 (amide group) cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz, δ): 1.18 (3H, t, $J = 7.2$ Hz, H-21), 1.45 (H, m, H-2β), 1.50 (H, dd, $J = 14.3$, 6.4, H-12α), 1.65 (H, ddd, $J = 13.7$, 13.7, 4.8 Hz, H-3β), 1.80 (H, dd, $J = 16.2$, 5.1 Hz, H-15β), 1.87 (H, m, H-3α), 1.89 (H, ddd, $J = 15.3$, 12.3 5.3 Hz, H-12β), 2.07 and 2.08 (3H, each, s, 2 × $OCOCH_3$), 2.10 (H, d, $J = 2.5$ Hz, H-5), 2.12 (H, m, H-2α), 2.12 (H, ddd, $J = 11.9$, 6.6, 6.6 Hz, H-10), 2.45 (H, dd, $J = 6.9$, 5.6 Hz, H-13), 2.56 (H, dd, $J = 16.3$, 9.0 Hz, H-15α), 3.02 (H, dd, $J = 6.4$, 4.7 Hz, H-9), 3.07 (H, dq, $J = 14.0$, 7.1 Hz, H-20B), 3.16 (H, dd, $J = 9.0$, 5.1 Hz, H-16α), 3.36, 3.40 and 3.42 (3H each, s, 3 × OMe), 3.39 (H, d, $J = 3.1$ Hz, H-17), 3.62 (H, t, $J = 4.6$ Hz, H-14β), 3.78 (H, s, H-6α), 4.17 (H, dq, $J = 14.0$, 7.2 Hz, H-20A), 4.31 (H, d, $J = 12.0$ Hz, H-18B), 4.62 (H, d, $J = 12.0$ Hz, H-18A), 4.90 (H, dd, $J = 9.7$, 7.7 Hz, H-1β); 13C NMR ($CDCl_3$, 125 MHz) 12.4 (q, C-21), 20.8 (q, CH_3-CO), 21.6 (q, CH3-CO), 26.8 (t, C-2), 28.2 (t, C-12), 29.8 (t, C-3), 33.1 (t, C-15), 36.8 (d, C-13), 42.6 (d, C-10), 43.4 (t, C-20), 45.2 (d, C-9), 45.5 (s, C-4), 49.2 (s, C-11), 49.9 (d, C-5), 56.6 (q, C-6′), 58.0 (q, C-14′), 58.7 (q, C-16′), 63.2 (d, C-17), 66.0 (t, C-18′), 75.8 (d, C-1), 77.0 (s, C-8), 82.1 (d, C-16), 82.8 (d, C-14), 86.2 (s, C-7), 91.8 (d, C-6), 169.7 (s, CH_3-O*C*O), 169.7 (s, CH_3-O*C*O), 170.3 (s, C-19); EIMS *m/z* $[M]^+$ 551 (12), 536 (68), 534 (32), 533 (100), 519 (18), 518 (36), 508 (10), 491 (18), 490 (14), 476 (22), 474 (28), 473 (17), 462 (14), 461 (10), 460 (19), 459 (10), 444 (12), 433 (25), 432 (94), 431 (11), 430 (15), 418 (25), 416 (12), 414 (26), 402 (11), 400 (20), 368 (11), 358 (14), 328 (12), 326 (12), 91 (19), 85 (20), 75 (48), 71 (31), 58 (12), 57 (14) and 55 (11). HREIMS *m/z* $[M]^+$ 551.2746, calculated for $C_{28}H_{41}NO_{10}$, 551.2730.

Insect Bioassays. S. littoralis and *L. decemlineata* were reared on artificial diet and potato foliage, respectively, and maintained at 22 + 1°C, >70% relative humidity with a L/D photoperiod of 16:8 hr in a growth chamber.

Feeding Choice Assays. These experiments were conducted with newly emerged fifth-instar *S. littoralis* and adult *L. decemlineata*. Percent feeding inhibition (% FI) was calculated as described in Reina et al. (2001). Compounds with an FI >70% were tested in a dose–response experiment to calculate their relative

potency (EC_{50} values, the effective dose for 50% feeding reduction), which was determined from linear regression analysis (% FI on log dose).

Oral Cannulation. This experiment was performed with preweighed newly molted *S. littoralis* L6-larvae as previously described (Reina et al., 2001). An analysis of covariance (ANCOVA) on biomass gains with initial biomass as covariate (covariate $P > 0.05$) showed that initial insect weights were similar among all treatments. A second ANCOVA analysis was performed on biomass gains with food consumption as covariate to test for postingestive effects (Raubenheimer and Simpson, 1992; Horton and Redak, 1993).

Hemolymph Injection. DMSO solutions of the test compounds (10 μg/insect) were injected into twenty adult *L. decemlineata* beetles as described in Reina et al. (2001). Beetle mortality was recorded up to 3 days after injection. Percent mortality was analyzed with contingency tables and corrected according to Abbott (1925).

Trypanocidal Activity. This activity was assayed on epimastigote forms of *T. cruzi*, Y strain, as described in González-Coloma et al. (2002a).

Cytotoxicity. Sf9 cells derived from *S. frugiperda* pupal ovarian tissue (European Collection of Cell Cultures, ECCC) and Mammalian Chinese hamster ovary cells (CHO, a gift from Dr Pajares, I. C. Biomédicas, CSIC) were grown as previously described (González-Coloma et al., 2002a). Cell viability was analyzed by an adaptation of the MTT colorimetric assay method (Mossman, 1983). The active compounds were tested in a dose–response experiment to calculate their relative potency (LD_{50} values, the effective dose that to give 50% cell viability) and was determined from linear regression analysis (% cell viability on log dose).

RESULTS AND DISCUSSION

Antifeedant effects of the test compounds were structure- and species-dependent (Table 1). Overall, *L. decemlineata* (CPB) responded to a larger number of compounds than *S. littoralis* (67 and 46%, respectively), according to their different feeding adaptations. The most active CPB antifeedants were compounds **9** and **33** (EC_{50} <0.2) followed by **12**, **27**, **7**, **8** (EC_{50} <0.5), **18**, **13**, **21**, **24**, and **6** (EC_{50} <1). This insect showed a moderate response to atropine. *S. littoralis* showed the strongest response to **24**, followed by **18** > **19** > **29** (EC_{50} <3) (Table 1). None of the model substances affected the feeding behavior of this insect.

Among the insect toxins, 50% and 19% of the tested compounds significantly increased CPB mortality or negatively affected *S. littoralis* larval performance, respectively (Table 1), indicating species-dependent tolerance to these alkaloids. The most toxic compound to CPB was aconitine (**1**, 100% mortality), followed by **10** (% mortality >60), **14**, **17**, **19**, **25**, **30** (% mortality >45), **12**, **13**, **29**, **33**, **36**, **38**, and **40** (% mortality >30). All the moderate toxicants plus compound **30** (% mortality >30) were behavioral antifeedants in choice tests, indicating that they

TABLE 1. EFFECTIVE ANTIFEEDANT DOSES AND MORTALITY[a] OF NORDITERPENE ALKALOIDS ON ADULT *L. decemlineata*

		L. decemlineata		*S. littoralis*		Sf9	
Compound	Type	EC_{50} ($\mu g/cm^2$)	%M	EC_{50} ($\mu g/cm^2$)	ΔB	ΔI	LD_{50} ($\mu g/ml$)
Atropine		7.38 (1.53, 35.82)	nt	>50	99	98	>100
Anabasine		>50	nt	~60	85	80	>100
Eserine		~60	nt	>50	54**	58**	>100
1	Aconitine	>100[b]	100	32.3 (19.6, 45.0)[b]	34**	67**	>100
2		>50	0	≈50	94	84**	>100
3		>50	8	8.29 (8.17, 8.42)	99	99	>100
4		2.57 (0.44, 14.88)	0	5.37 (3.14, 45.47)	91	90	>100
5		≈50	15*	≈50	90	90	>100
6		0.99 (0.97, 1.02)	nt	>50	111	99	>100
7		0.44 (0.20, 0.97)	32*	>50	89	96	>100
8		0.42 (0.40, 0.43)	4	≈50	26**	70**	>100
9		0.11 (0.01, 1.72)	0	21.84 (4.32, 51.27)	90	112	30.39 (25.23, 36.61)
10	Lycoctonine	>50	61*	>50	45**	71**	>100
11		6.00 (1.96, 18.42)	0	>50	69**	112	>100
12		0.23 (0.04, 1.29)	34*	>10	79**	94	>100
13		0.60 (0.18, 2.01)	37*	>50	111	104	29.17 (21.40, 40.67)
14		≈50	47*	17.99 (17.70, 18.30)	78**	95	0.38(0.22, 0.66)
15		$12.53\ (2.71, 57.85)_{ns}$	1	>50	94	90	>100
16		≈50	21*	9.86 (4.83, 20.16)	105	115	>100
17		nt	47*	nt	98	101	>100
18		0.54 (0.53, 0.56)	11	0.84 (0.82, 0.86)	107	104	>100
19		>50	41*	1.51 (1.48, 1.51)	106	99	14.88 (5.02, 44.08)
20		2.22 (0.96, 5.08)	0	>50	89	93	>100
21		0.66 (0.64, 0.68)	7	5.29 (5.18, 5.42)	91	96	>100
22		13.02 (12.77, 13.28)	0	9.31 (9.09, 9.91)	100	128	>100
23		1.11 (0.34, 3.58)	1	3.53 (3.46, 3.60)	92	97	32.37 (17.20, 58.09)

TABLE 1. CONTINUED

Compound	Type	*L. decemlineata*		*S. littoralis*		Sf9	
		EC_{50} ($\mu g/cm^2$)	%M	EC_{50} ($\mu g/cm^2$)	ΔB	ΔI	LD_{50} ($\mu g/ml$)
24		0.84 (0.82, 0.85)	24*	0.42 (0.41, 0.44)	96	95	>100
25		nt	47*	nt	80	87	>100
26		>50	0	>50	115	105	>100
27		0.29 (0.04, 1.82)	14*	5.63 (5.54, 5.72)	100	111	>100
28		nt	27*	nt	107	103	>100
29		2.97 (2.94, 3.02)	32*	2.72 (2.68, 2.76)	82	91	>100
30		2.78 (2.72, 2.85)	47*	17.77 (5.88, 53.66)	90	93	>100
31		11.93 (3.14, 45.47)	0	>50	97	103	>100
32	Gadesine	≈60	1	13.61 (13.42, 13.81)	86	87	>100
33		0.13 (0.01, 1.42)	34*	>50	110	109	>100
34		1.49 (0.31, 7.24)	11	14.29 (8.50, 24.08)	82	93	>100
35		6.36 (2.16, 18.76)	0	>50	88	88	>100
36		12.2 (20, 73.82) ns	31*	nt	99	96	18.89 (9.36, 38.17)
37	Miscellaneous	4.43 (1.54, 12.73	12	>50	101	102	>100
38		3.31 (1.10, 9.94)	37*	11.79 (11.70, 11.89)	97	98	1.83 (1.18, 2.83)
39		2.36 (0.47, 11.80)	25*	5.38 (1.43, 20.37)	91	88	>100
40		1.92 (0.66, 5.54)	31*	≈50	106	96	6.27 (3.26, 12.05)
41		>50	nt	>50	61**	61**	29.45 (17.46, 49.67)
42		3.62 (3.54, 3.69)	nt	3.33 (1.07, 10.39)	118	112	>100
43		10.92 (10.75, 11.10)	nt	>50	76**	69**	>100

Note. Consumption (ΔI) and Biomass Gain (ΔB) of orally injected *S. littoralis* L6 larvae is expressed as percent of the control. Cytotoxic effects on *S. frugiperda* Sf9 cells. nt, not tested (insufficient compound available); ns, not significant dose–response relationship, $P > 0.05$.

[a] %M, 72 hr data corrected according to Abbot, 1925.

[b] From González-Coloma et al., 1998.

* Significantly different from the control, $P < 0.05$, contingency table analysis; ** Significantly different from the control, $P < 0.05$, LSD test.

act at both the peripheral and central nervous system, and suggesting a negative correlation between antifeedant and toxic effects on CPB.

Orally injected *S. littoralis* larvae were negatively affected by **1**, **8**, **10**, **11**, **12**, **14**, **41**, **43**, and eserine with varying degrees. A covariance analysis (ANCOVA1) of food consumption (ΔI) and biomass gains (ΔB) with initial larval weight (BI) as covariate (covariate $P > 0.05$) was performed to test for significant effects of the test compounds on these variables. An additional ANOVA analysis and covariate adjustment on ΔB with ΔI as covariate was performed for the compounds that significantly reduced ΔB.

Alkaloids **11**, **12**, and **14** moderately reduced ΔB without decreasing ΔI (ANCOVA1 $P < 0.05$ for ΔB). Treatment effects on ΔB did not disappear with covariance adjustment, indicating that these compounds were postingestive toxins without delayed antifeedant effects. Compounds **1**, **8**, **10**, **41**, **43**, and eserine significantly decreased ΔI and ΔB (ANCOVA1 $P < 0.05$ for ΔB and ΔI). Treatment effects on ΔB disappeared with covariance adjustment except for cardiopetaline (**8**). Therefore, **1**, **10**, **41**, **43**, and eserine had postingestive antifeedant effects, while **8** also had further toxic action. Similar effects of neuroactive β-carboline alkaloids on *Trichoplusia ni* growth and consumption have been attributed to their interference with neurochemical mechanisms regulating food intake (Heinz et al., 1996). From among these toxins, **1** and **14** also altered the feeding behavior of *S. littoralis* in choice tests.

A few compounds (18%) randomly distributed among the chemical classes had selective cytotoxic effects to insect-derived Sf9 cells (none of these compounds was cytotoxic to mammalian CHO cells) and did not increase *T. cruzi* mortality (data not shown). This cytotoxicity indicates a mode of action other than neurotoxic. Compound **14** was the most active (LD_{50} <0.5), followed by **38** (LD_{50} <2), **40** (LD_{50} <7), **19**, **36** (LD_{50} <20), **13**, **41**, **9**, and **23** (LD_{50} <35). Some of these cytotoxic compounds were also toxic to CPB (**13**, **14**, **19**, **36**, **38**, **40**) and/or *S. littoralis* (**14**, **41**); therefore, their insecticidal effects could be the result of neurotoxicity and/or cytotoxicity. The lack of insect toxicity of **9** and **23** could be the result of metabolic detoxification or excretion. Hepatic P450 enzymes in mammals apparently do not metabolize some of these compounds (Panter et al., 2002), suggesting that other enzymes or an excretion mechanism could be involved in insect detoxification of norditerpenoid alkaloids. This is the first report of the cytotoxic effects of norditerpenoid alkaloids on insect cells. The cytotoxic mode of action is not neurotoxic, and the selectivity between insect and mammalian cells might be related to membrane factors.

To facilitate study of the SAR, the alkaloids have been grouped into aconitine-, lycoctonine-, gadesine-, and miscellaneous-type compounds (Figures 1–4).

SAR of the Aconitine-Type. All CPB antifeedants (**9**, **8**, **6**, **4**) lacked the C-14 benzoyl, and the most active ones (**9**, **8**) lacked oxygenated substitutions at C-16. The most active *S. littoralis* antifeedants (**4**, **3**) had an OCH_2CH3 group at C-8 and

a benzoyl ester at C-14 (**3**), indicating species-dependent molecular selectivity in relation with these substituents. Acetylation of the strong insect toxin **1** resulted in the loss of this activity (compound **2**). Therefore, hydroxylation at C-3 plays an important role in the insect toxic effects of the aconitine-type. Similarly, acetylation of C-1 and C-14, as in **9**, resulted in the loss of *S. littoralis* toxicity compared to **8**, while conferring cytotoxicity.

SAR of the Lycoctonine-Type. The most active CPB antifeedants (**12**, **27**, **18**, **13**, **21**, **24**, **23**) and toxicants (**10**, **14**, **17**, **25**) had oxygenated substituents (OH/OAc) at C-14 and a wide range of substitutions at C-18, indicating that this insect did not have a strong molecular selectivity for this chemical-type. *S. littoralis* was mostly sensitive to compounds with a βOMe substituent at C-6, and the most active antifeedants (**24**, **18**, **19**) were also acetylated at C-14. Deacetylation/methylation at C-14 and hydroxylation at C-18 lowered this antifeedant activity (**29**, **23**, **21**, **27**, **22**). A lack of substitution at C-18 (**10**, **11**, **12**) seemed to be the most important characteristic for this chemical class to negatively affect *S. littoralis* larval performance, except for **31** with a bulky OBz group at C-14. Compound **14**, a C-6 α-epimer with a C-14 hydroxy, was the most cytotoxic alkaloid. This activity was lower with an OAc at C-14 (**19**) and OH at C-18 (**13**), and lost with an OAc/OMe at C-14 (**15**, **16**, **20**).

SAR of the Gadesine-Type (1,19 epoxides). All structures were CPB antifeedants except for **32** with a benzoyl at C-14. The most important feature for this activity is the hydroxylation at C-18 (**33**, **34**) followed by hydroxylation at C-14 (**34**, **35**) and/or methylation at C-18 (**35**, **36**). Compound **32** was a moderate antifeedant to *S. littoralis*, supporting species-dependent molecular selectivity determined by the C-14 benzoyl group, previously shown for the aconitine-type. Compound **36** was toxic to CPB, *S. littoralis* larvae, and cytotoxic to Sf9 cells, while **33** was only toxic to CPB, suggesting that the C-18 methylation in **36** increased bioavailability for these targets.

SAR of the Miscellaneous-Type. The presence of a 7,8-methylenedioxy group in ajadelphinine (**37**), with a C-14 hydroxylation, gave a moderate-low CPB antifeedant effect. All the 3,4 epoxides (**38–40**) were moderate CPB and *S. littoralis* antifeedants with negative rank-correlation. These three compounds were also moderate CPB toxicants. Epoxides **38** and **40**, with an OMe substituent (at C-14 and C-18, respectively), were the second and third most cytotoxic compounds to Sf9 cells, indicating that the epoxide may play a role in this effect. Compound **41** acted as an *S. littoralis* toxin with moderate-low Sf9 cytotoxicity. Structurally-related compounds **20** and **22** were not cytotoxic. Therefore, the 19-oxo and the 1,18-diacetyl determined this action. Compounds **42** and **43** with a C(19) = *N* azomethine group had reduced CPB and *S. littoralis* antifeedant activity with respect to related compounds **18** and **29**. Compound **43** was a stronger *S. littoralis* toxicant than the structurally related **29**.

The action of norditerpenoid alkaloids on the voltage-dependent sodium channels can be separated into activators (alkaloids with a benzoyl substituent at C-14) with extremely high toxicity in mammals, and blockers (Friese et al., 1997). Among the Na^+ channel agonists are aconitine (**1**) and 3-acetylaconitine (**2**) (Seitz and Ameri, 1998), while several lycoctonine-type alkaloids (including lycoctonine, **26** and methyllycaconitine, **30**) are competitive antagonists at the muscular and/or insect nAcChR junction (Jennings et al., 1986; Dobelis et al., 1999). The intensity of the nAChRs inhibition by norditerpene alkaloids is structure-dependent. The active core is the lycoctonine skeleton. The methylsuccinylanthranoyl ester at C-18 and the quaternary amine are important factors of the neuromuscular blocking effect (see Panter et al., 2002). In addition, the C-14 functionalities, the pattern of oxygenation, and the electronic nature of the oxygen bearing functionalities appear to enhance potency (Kukel and Jennings, 1994; Hardick et al., 1996; Dobelis et al., 1999).

Neither the antifeedant nor the toxic activity of the compounds studied here followed the expected SAR based on their receptor binding activity. The C-14 benzoyl group of agonists **1** and **2**, and related compounds **3** and **32**, had no, or low, CPB and *S. littoralis* taste regulation, respectively, while aconitine (**1**) was a strong toxin to both insects. The C-18 methylsuccinylanthranoyl substituent in methyllycaconitine (**30**) did not cause potent antifeedant action (35% and 41% of the active compounds were more potent CPB and *S. littoralis* antifeedants, respectively, than **30**), in contrast to the C-18 benzoyl (**24**). In addition, their antifeedant effects did not correlate with toxicty. A lack of correlation between receptor binding activity and antifeedant/toxic effects might be related to compound bioavailability/penetrability. This activity pattern could also be related to the mode of action on nAChRs (agonists vs. antagonists).

Previous studies have shown that agonists of insect nAChRs were generally insecticidal (toxic), whereas antagonists, such as imidacloprid, were antifeedants (Nauen et al., 1999). However, there is no evidence of the direct link between antifeedant effects and antagonistic action of compounds on insect nAChRs.

GABA-mediated taste regulation has been proposed for chrysomelids and aphids (Mullin et al., 1997, González-Coloma et al., 2002b; Reina et al., 2002). However, given the structural diversity of plant natural products and the increasing evidence of peripheral neuroreception involved in insect taste regulation (Sanes et al., 1977; Bloomquist, 2001; Cohen et al., 2002), we propose a species-dependent multireceptor/channel mechanism for insect taste mediation tuned according to feeding adaptations and involving nAChRs among others.

In summary, we have demonstrated here that a wide array of norditerpenoid alkaloids act as insect antifeedants and toxicants, supporting their plant defense role. Potency did not parallel nAChR binding activity, but did correlate with the agonist/antagonist insecticidal/antifeedant model proposed for nicotininc insecticides.

This supports nAChR mediation in insect taste regulation, and opens a new field for insect control strategies. The selective cytotoxic effects of some structures suggest that these compounds can act on biological targets other than neuroreceptors with strong molecular selectivity as previously demonstrated for several alkaloids belonging to different chemical classes (Wink et al., 1998).

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SUPPLEMENTARY DATA

Experimentally updated spectroscopic data as described in Material and Methods Section and references for the test compounds are available.

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CREOSOTE BUSH (*Larrea tridentata)* RESIN INCREASES WATER DEMANDS AND REDUCES ENERGY AVAILABILITY IN DESERT WOODRATS (*Neotoma lepida*)

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Abstract—Although many plant secondary compounds are known to have serious consequences for herbivores, the costs of processing them are generally unknown. Two potential costs of ingestion and detoxification of secondary compounds are elevation of the minimum drinking water requirement and excretion of energetically expensive metabolites (i.e., glucuronides) in the urine. To address these impacts, we studied the costs of ingestion of resin from creosote bush (*Larrea tridentata*) on desert woodrats (*Neotoma lepida*). The following hypotheses were tested: ingestion of creosote resin by woodrats (1) increases minimum water requirement and (2) reduces energy available by increasing fecal and urinary energy losses. We tested the first hypothesis, by measuring the minimum water requirement of woodrats fed a control diet with and without creosote resin. Drinking water was given in decreasing amounts until woodrats could no longer maintain constant body mass. In two separate experiments, the minimum drinking water requirement of woodrats fed resin was higher than that of controls by 18–30% (about 1–1.7 ml/d). We tested several potential mechanisms of increased water loss associated with the increase in water requirement. The rate of fecal water loss was higher in woodrats consuming resin. Neither urinary water nor evaporative water loss was affected by ingestion of resin. Hypothesis 2 was tested by measuring energy fluxes of woodrats consuming control vs. resin-treated diets. Woodrats on a resin diet had higher urinary energy losses and, thus, metabolized a lower proportion of the dietary energy than did woodrats on control diet. Fecal energy excretion was not affected by resin. The excretion of glucuronic acid represented almost half of the energy lost as a consequence of

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resin ingestion. The increased water requirement and energy losses of woodrats consuming a diet with resin could have notable ecological consequences.

Key Words—Woodrats, creosote resin, secondary compounds, herbivores, water requirement, osmolarity, urine, metabolizable energy, glucuronic acid, detoxification.

INTRODUCTION

The potential consequences of ingestion of plant secondary metabolites (PSMs) are numerous. Herbivores consuming diets with PSMs may exhibit decreased digestibility, alteration of the central nervous system, and decreased reproduction among others (Freeland and Janzen, 1974; Haukioja, 1980; Meyer and Karasov, 1989; Belovsky and Schmitz, 1991; Bryant et al., 1992; Silverstein, et al., 1996). However, the effect of PSM ingestion on water balance of herbivores has been largely ignored (Dearing et al., 2001). Previous studies (Mangione et al., 2000; Dearing et al., 2001, 2002) implied that ingestion of PSMs increases water flux in desert herbivores. For example, desert woodrats increased water intake by 50–76% with increasing levels of dietary toxin (Mangione et al., 2000). If this increase in water consumption is obligatory, it doubles the normal minimum requirement of preformed water for desert woodrats consuming dietary toxins (Karasov, 1989). The consequences of an increase in water consumption could have significant implications for diet selection by herbivores whose only source of preformed water is that in plants.

Besides affecting water balance, PSMs may impact energy availability. There are three ways that PSMs may reduce energy availability. First, they can dilute the available energy in food, as they typically cannot be used as a source of energy (Jakubas et al., 1993b). Second, they may retard the extraction of available food energy. Decreased energy extraction can occur if PSMs inhibit digestive enzymes or bind to food components such that they cannot be digested or absorbed, as may occur for tannin–protein complexes (Glick and Joselyn, 1970a,b; Lindroth et al., 1984; Robbins et al., 1987a,b, 1991). Lastly, they can cause postingestive energy loss through increased energy metabolism during detoxification (Iason and Murray, 1996), excretion of conjugation molecules (Dash, 1988; Remington, 1990; Guglielmo et al., 1996), or excretion of endogenous materials such as salivary proteins (Dietz et al., 1994; McArthur et al., 1995). These effects are measurable as reductions in the digestibility or metabolizability of energy. Regardless of the exact mechanism, reductions in dietary energy availability can compromise the energy balance of herbivores ingesting PSMs.

In this study, we examined how ingestion of plant toxins affects energy availability and water requirements of a herbivorous rodent. We focused on woodrats (*Neotoma lepida*) from the Mojave desert because they feed on creosote (*Larrea tridentata*), renowned for its toxic secondary chemistry (Cameron and Rainey,

1972; Karasov, 1989). Leaves of creosote contain between 10 and 25% phenolic resin (by dry mass), 40% of which is NDGA (nordihydroguaiaretic acid), a well-documented toxin to mammals (Grice et al., 1968; Goodman et al., 1970; Mabry, et al., 1977; Sheikh et al., 1997). The remainder of the resin is a complex mixture of partially O-methylated flavones and flavonols (Rhoades, 1977). Water content of creosote in the Mojave desert ranges from about 0.5 to 1.0 ml/g dry mass, depending on season, with water content being highest in spring following winter rains and lowest in fall just prior to winter rains (Nagy et al., 1976; Schmidt-Nielsen, 1979; Karasov, 1989).

We tested the hypothesis that ingestion of PSMs increases the minimum water requirement of herbivores. We predicted that herbivores eating a diet with PSMs would require more drinking water to maintain body mass than those consuming the same diet without toxins. We investigated the following potential causes of increased water loss as a result of dietary PSMs: i) increase in osmotic load due to an increase in either food intake or organic acid load as a result of detoxification and excretion of PSM metabolites in urine; ii) a diuretic effect of the resin resulting in higher urine water content; iii) an increase in fecal water content; or iv) an increase in evaporative water loss rate as might occur if metabolic rate increased. Components of water influx and efflux were determined in desert woodrats drinking the minimum water necessary for body mass maintenance in the presence and absence of creosote resin in the diet.

We also tested whether creosote resin decreases energy digestibility and metabolizable energy. In previous studies, Meyer and Karasov (1989) demonstrated that the phenolic resin from creosote leaves had no effect on either dry matter digestibility or nitrogen digestibility. Although this result suggests that creosote resin does not function as a digestibility reducer as proposed by Rhoades (1977), energy digestibility was not measured. Also, the resin might increase energy losses postabsorption, due to detoxification of PSMs. Woodrats significantly increase the amount of detoxification products (e.g., glucuronides and sulfates) in the urine after the ingestion of creosote resin (Mangione et al., 2001). Losses of glucose, glycine, and sulfate moieties used in conjugation could reduce the energy available to woodrats. We predicted that metabolizable energy would be lower in animals fed resin-treated diets than in control diets. We compared the energy fluxes of desert woodrats on diets with and without creosote resin.

METHODS AND MATERIALS

Field Site and Sample Collection. Desert woodrats were trapped at Beaver Dam, Grand Co., UT (37°06′N, 113°58′W). The vegetation at Beaver Dam was primarily composed of creosote bush, black brush (*Coleogyne ramosissima*), Joshua

tree (*Yucca brevifolia*), desert almond (*Prunus fasciculata*), and, less commonly, cholla (*Opuntia spp.*).

Woodrats were captured between April 10 and 12, 1996, using Tomahawk and Sherman live traps baited with peanut butter and oats (see Mangione et al., 2000, for details). All woodrats were transported to the animal facility at the University of Utah and kept in quarantine closets for 2–5 m while they were determined to be free of Sin Nombre hantavirus (Dearing et al., 1998) and then were transported to the Department of Wildlife Ecology, University of Wisconsin, Madison, WI. Ten kilograms (wet mass) of creosote leaves were collected on April 11, 1996, at Beaver Dam from a stand of creosote near the trapping areas. A mixture of young and mature foliage was clipped from stems not bigger than 0.3 cm in diam. The foliage was placed onto dry ice and kept at -20°C until the resin was extracted.

Animal Housing and Diet Preparation. All feeding trials were conducted at the University of Wisconsin – Madison. The experimental protocols were approved by the Research Animal Resources Center (RARC), University of Wisconsin – Madison. Woodrats were housed in metal cages ($47 \times 30 \times 21$ cm) with screened bottoms. Animals were provided with cotton bedding and a ceramic bowl in which to nest. The room was kept at 21°C and 65% relative humidity on a 12:12 L/D cycle. When not involved in experiments, woodrats were provided water *ad libitum*, high-fiber rabbit chow *ad libitum* (Harlan Teklad 8630, Wisconsin), and occasionally apples.

Resin from creosote leaves was extracted by soaking the leaves and stems in diethyl ether (1 part wet leaves: 5 parts solvent) for 45 min. The ether solution was filtered through Whatman N$^{\circ}$4 filter paper, poured into beakers in a water bath at 40°C, and the ether removed by boiling for 2–4 hr until the filtrate reached a dense and viscous consistency. The resin was stored at -25°C for up to 7 m prior to use.

The resin diet was prepared by adding 3.7 g of resin to 25 ml of 95% ethanol per 100 g of ground rabbit chow. Results from other experiments indicated that this concentration was near the maximum tolerable amount that adult woodrats would ingest without losing $>10\%$ body mass (Mangione et al., 2000). The resin/ethanol solution and the chow were thoroughly mixed, and dried overnight (room temperature) until the ethanol was evaporated (confirmed gravimetrically). Control and resin-treated diets were pelleted and stored in the freezer until used. Because both heat and water can alter the properties of phenolics in diets (Price et al., 1980) and alter diet palatability (Dietz et al., 1994; Lindroth et al., 1984), we blanketed the pellet machine with a plastic bag filled with crushed ice to minimize heating during pelleting. The amount of water used to make the pellets (both control and resin-treated diets) was minimized by adding 7–10% (volume solution/mass of food) of a 60% ethanol solution. The final pellets were dried overnight to constant mass at room temperature and then stored in the freezer until use.

EXPERIMENTAL DESIGN

Water Requirement. Two experiments were performed to determine whether resin consumption resulted in an increase in minimum water requirement and to ensure that the urine collection method used in the first experiment yielded reliable results.

Experiment 1. Minimum water requirement of woodrats ($N = 6$) was determined for each of the following sequential treatments; resin-free diet, resin-containing diet, and resin-free diet (hereafter called control #1, resin, and control #2, respectively). Woodrats were given water and food *ad libitum* for 1 m between treatments. To determine the minimum water requirement, the water ration (measured daily to ± 0.1 g; density of water = 1 ml/g) was reduced gradually over a 3-wk period until the woodrat could not stabilize its body mass at a lower water ration. Specifically, at the beginning of each trial, water was offered *ad libitum*, and on the 4th day it was reduced to 60% of *ad libitum*. When a reduction in body mass (measured daily to ± 0.1 g) was detected, the ration offered the previous day was maintained until body mass stabilized for at least 2 d. At this point, a new reduction (20%) of water was imposed. If a woodrat lost body mass (2% or more) for more than 4 d, the ration was increased slightly (<0.5 ml/d) and body mass was checked again daily. When body mass stabilized for at least 2 d, the ration was reduced again to test if the woodrat would lose body mass and to ensure that the ration was, in fact, the minimum water required to maintain body mass. Considering the sensitivity of our measures of water and animal mass (± 0.1 g), we think that our precision in measuring minimum water requirement is <0.5 g/d.

A detailed example showing body mass fluctuations with water offered over time is given in Figure 1. In this example, on day 4, available water was decreased from 12 to 6.5 ml. This resulted in a rapid decline in body mass of approximately 5.5% from initial. Therefore, on d 5, available water was increased to 7 ml to reduce the continual weight loss. By d 9, body mass was reasonably stable so the available water was decreased to $\sim$5.4 ml/d. This decrease caused a continual decline in body mass, necessitating two increases of 0.5 ml/d on ds 12 and 14 to stabilize body mass. During these periods, increments or reductions in water of only 0.1 ml/d were enough to cause parallel changes in body mass. Thus, the minimum water requirement for this animal was determined to be 5.8 ml/d.

Once at minimum water, components of water influx and efflux were measured for 7 d (see bracket on Figure 1). During this period, food, water intake, and body mass were measured daily. Woodrats were restricted to a portion ($16 \times 19 \times 20$ cm) of their cage that permitted the separate collection of urine and feces. Pilot experiments showed that there were no differences in food intake when woodrats were in either section of the cage. Urine, uncontaminated by food

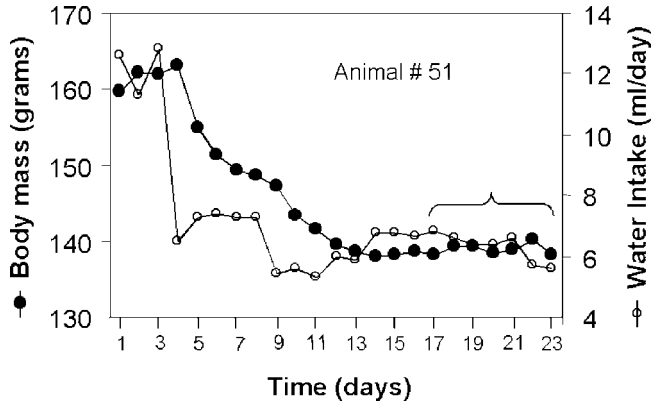

FIG. 1. A representative example of the relationship between available water and body mass (from experiment 1). Bracket shows the period for which water intake, food intake, body mass, and fecal output were measured daily for calculations.

and feces, was separately collected in plastic vials on ice to minimize bacterial growth and evaporation. All feces and urine were collected and dried. Feces were dried in the oven at 65°C to constant mass. Urine samples used for energy content quantitation were lyophilized. For calculation of total urine volume excreted, the small amounts of dry urine that adhered to the funnels and the bottom of the cage were collected with a hot distilled water rinse and added to the rest of the liquid urine collected. Fecal and urinary water losses were calculated as the product of the dry mass excreted multiplied by the respective water content measured in freshly collected samples. To determine the fecal water content, fresh feces were collected periodically during the night and day throughout experiment 1. Metabolizable energy of dry mass ingested was calculated from the results shown below for metabolizable energy coefficients. Evaporative water loss was calculated at the end of the experiment by measuring rates of body mass change during the last 12 hr of a 24-hr fast. In a fasting mammal whose respiratory quotient is approximately 0.7, mass change due to respiratory loss of CO_2 is approximately balanced by a greater retention of O_2, which is incorporated into metabolic water. Thus, any mass decrease is due largely to evaporation or urinary and fecal loss. Urine or feces were collected at 3-hr intervals in a tared container with mineral oil. This period gives enough time to obtain samples, but not enough to allow feces to disintegrate in the oil.

Experiment 2. A month and a half after completion of experiment 1, a second experiment was performed to corroborate the findings on water requirements. This experiment used a crossover design to block more effectively for any potential effect of time, and to test for possible overlooked urinary losses in the collection

system used in experiment 1. Initially, 6 woodrats were randomly divided into 2 groups, control and resin. Minimum water required was determined for each group following the procedure described in experiment 1. Woodrats were allowed to recover for approximately a month before being switched to the other treatment. At the end of each trial, food intake rate, body mass, and urine output were measured daily for 4 d. To minimize possible evaporation or losses of water from the system, urine was collected under mineral oil into a tray located 1 cm beneath the cage. Urine volume was measured in a graduated cylinder. Feces were collected in nylon mesh located 1 cm beneath the steel mesh of the cage floor. Even though an effort was made to collect fresh feces to determine the water flux by this route (as in experiment 1), the samples were incomplete. At the end of the first run in experiment 2, one of the woodrats was euthanized because it abruptly lost ~37% of body mass. Thus, the final sample size was 5 animals.

In both experiments, water intake was measured by weighing water bottles and correcting for both evaporation and spillage to the nearest 0.1 g. Spillage, ~0.3 ml/d, was determined by measuring the volume of water that dripped daily into a container with mineral oil. Dry matter intake was measured as the difference between what was given and what remained each day after the samples were dried at 50°C for 3 d. Water intake from food was calculated as the product of food intake and the water content of the pellets. Content of water in urine was measured for each animal in experiments 1 and 2 by drying an aliquot of urine to constant mass. Urine osmotic pressure was measured with a Wescor 510B Vapor Pressure Osmometer. When urine concentration exceeded the capacity of the osmometer, samples were diluted 1:5 with distilled water and remeasured. This may lead to 10–25% overestimation of the concentration because the ionization of salts increases with dilution (Karasov, 1989; Sweeney and Beuchat, 1993). In fact, we found an average overestimation of 24% for both controls and treatments when we compared 1:5 vs. 1:2 dilutions of urine (data not shown). However, these overestimates should not greatly confound the comparisons made within our study because dilutions were applied without bias to the two treatment groups.

Energy Availability. Energy losses in feces and urine were measured by bomb calorimetry using urine and fecal samples from experiment 1. Data on body mass, food and resin intake, fecal production, and urine production were measured for days 5, 6, and 7.

Energy Content of Feces and Urine. Urine and fecal samples from days 5, 6, and 7 of each of the treatments in experiment 1 were frozen at −25°C until analysis. Because the animals were in steady state, one sample per animal (days 5, 6, and 7 pooled) of feces and of urine was analyzed in duplicate. Feces were ground in a Wiley mill (1-mm screen). Before urine samples were pooled, they were lyophilized to constant mass, then, the same amount of dried urine from each day was weighed. The 3 samples were mixed and ground in a mortar with liquid nitrogen. Pooled samples of urine were used to make the pellets for

bomb calorimetry. The pellets were lyophilized again because the urine absorbed water after being ground and pelleted. Lyophilized urine pellets were stored in a vacuum-sealed desiccator until used. Fecal pellets were redried at 50°C, 24 hr before they were bombed. Energy content of urine and feces were measured using a Phillipson Microbomb Calorimeter (Gentry Instruments) with benzoic acid as a standard.

Digestibility and metabolizability of food energy were calculated using the equations:

$$\text{ADEC} = (\text{Ge}_i Q_i - \text{Ge}_f Q_f)/\text{Ge}_i Q_i \quad \text{and}$$

$$\text{MEC} = (\text{Ge}_i Q_i - \text{Ge}_f Q_f - \text{Ge}_u Q_u)/\text{Ge}_i Q_i$$

where ADEC and MEC are apparent digestible and apparent metabolizable energy coefficients (unitless proportions of energy), respectively; Q_i, Q_f, and Q_u are dry matter intake, fecal output, and urine output in grams/d; Ge_i, Ge_f, and Ge_u are the energy contents of food, feces, and urine in kJ/g (Robbins, 1993). Both ADEC and MEC were expressed as percentage of energy intake in Figure 6a and b).

Data Analysis. Components of water influx and efflux from experiment 1 (drinking water, dry matter intake, water content of the urine, fecal output, water content of feces water efflux from feces, and evaporative water loss) were compared using a repeated measures ANOVA with treatment as the repeated measure. Differences between treatments were examined with *post hoc* contrasts. Drinking water, dry matter intake, water content of the urine, and water flux from urine during experiment 2 were compared between treatments using repeated measures ANOVA with one grouping factor (the sequence in which the diet was offered, either control–resin or resin–control). Because one of the animals in experiment 2 died during the first trial, sample size used in the analysis was 5.

The effect of treatments on ADEC and MEC was tested with repeated measures ANOVA (treatments = control 1, resin, and control 2 as the repeated measures). Proportions were arcsine transformed before statistical analysis. This analysis is the most appropriate one because the samples are not independent from each other.

Because there is controversy regarding the comparison of ratio-based assimilation efficiencies (Beaupre and Dunham, 1995), we also tested for an effect of treatment on fecal energy and urinary energy excretion, using the ANCOVA method of Beaupre and Dunham (1995). We regressed fecal energy excretion rate as the dependent variable with food energy intake as the covariate. Urinary energy excretion was regressed against digestible energy intake as the covariate. In both analyses of ANCOVA, the interaction terms were not included in the models because they were not significant, so we tested only for possible differences

between treatments. Both repeated measures ANOVA and ANCOVA were done using (SYSTAT version 5.03; Wilkinson, 1992). In all cases, values are expressed as mean ± one standard error (N = number of woodrats).

RESULTS

Water Requirement. There was no consistent effect of resin ingestion on body mass of woodrats. In experiment 1, the body mass of woodrats eating resin (118.9 ± 11.5 g) was higher than the body mass of woodrats during the second control period (113.5 ± 10.5 g), but not the first control period [118.8 ± 11.7 g; $F(2, 10) = 5.21$, $P = 0.02$]. In experiment 2, there was no difference in body mass between woodrats consuming resin (114.7 ± 7.0 g) or control diet [Control: 116.5 ± 8.3 g; $F(1, 4) = 1.04$, $P = 0.36$].

The effect of resin on minimum water requirement was consistent across both experiments. The minimum requirement for drinking water was higher in woodrats fed resin than in controls, by 18% in experiment 1 [Control 1: 5.5 ± 0.25 ml/d; Resin: 6.5 ± 0.8 and 5.5 ± 0.33 ml/d; $F(2, 10) = 3.81$, $P = 0.059$], and by 30% in experiment 2 [Control: 5.6 ± 0.2 ml/d and Resin: 7.3 ± 0.5 ml/d; $F(1, 3) = 19.22$, $P = 0.022$]. There were no significant differences in food intake between control and resin treatments in either experiment [experiment 1: $F(2, 10) = 1.94$, $P = 0.193$, and experiment 2: $F(1, 3) = 1.47$, $P = 0.31$; Figure 2].

Fecal water loss was higher in woodrats eating resin diets [$F(2, 10) = 5.67$, $P = 0.023$], apparently because of the combination of small increases in both fecal water content [$F(2, 10) = 2.24$, $P = 0.15$] and fecal dry matter flux [$F(2, 10) = 2.86$, $P = 0.104$; Figure 3].

Urine water fluxes were not affected by resin in either experiment [experiment 1: $F(2, 10) = 2.25$, $P = 0.15$, and experiment 2: $F(1, 3) = 0.35$, $P = 0.59$]. Urine water content was not elevated in resin-fed woodrats in either experiment [experiment 1: $F(2, 10) = 3.09$, $P = 0.09$, and experiment 2: $F(1, 3) = 0.021$, $P = 0.89$; Figure 4]. Urine osmotic pressure was lower for woodrats fed the resin-treated diet. In experiment 1, woodrats ingesting resin had lower osmotic pressure than one of the two control groups [$F(2, 10) = 14.9$, $P = 0.001$]. Woodrats fed resin also had lower urine osmolarity than woodrats fed the control diet in experiment 2 [$F(1, 3) = 966.36$, $P < 0.001$; Figure 4]. In experiment 1, evaporative water loss varied among trials but there was no consistent effect of resin [control 1: 2.8 ± 0.2 g/d, resin: 3.6 ± 0.3 g/d, control 2: 4.3 ± 0.3 g/d; $F(2, 10) = 5.88$, $P = 0.02$].

Energy Availability. Fecal energy excreted per day was not different between treatments [$F(2, 14) = 0.27$, $P = 0.76$] but increased with food energy intake [$F(1, 14) = 142.5$, $P < 0.001$; Figure 4a]. Urinary energy excretion was higher (~40%) for woodrats eating the resin-treated diet [$F(2, 14) = 8.7$, $P = 0.004$]

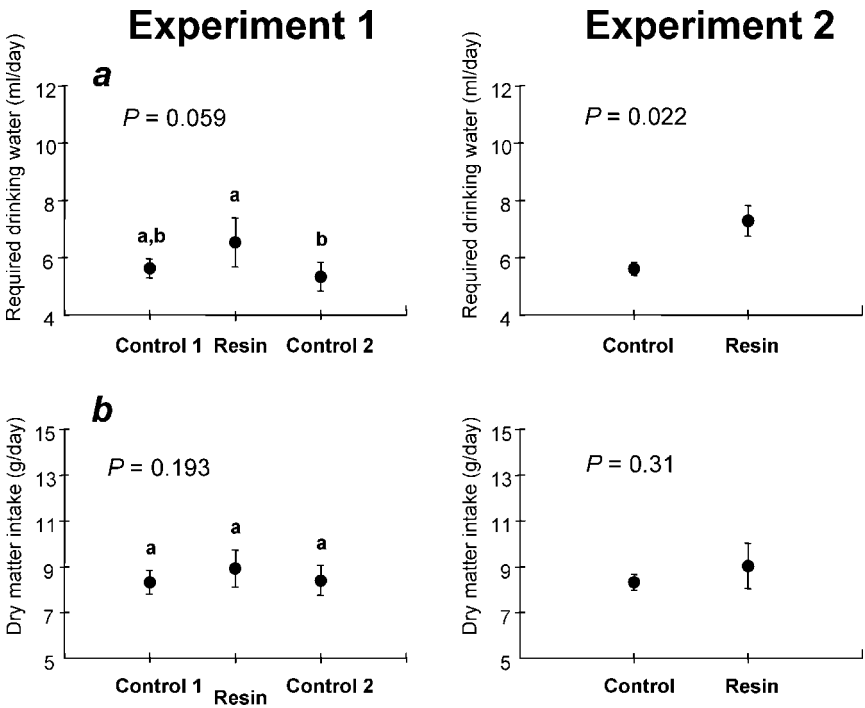

FIG. 2. Variation of required water (a) and dry matter (b) intake by woodrats consuming control and resin diets in experiments 1 and 2. Values are expressed as mean plus or minus one standard error. Means with the same lowercase letter indicate no significant difference. Differential contrasts were used to test for difference between continuous treatments in experiment 1 and special contrasts to test for differences between control 1 and control 2. For experiment 2, P values represent the overall significance for the repeated measures ANOVA with one grouping factor (diet sequence and either control-resin or resin-control).

and increased with digestible energy intake [$F(1, 14) = 5.52$, $P = 0.034$; Figure 4b].

The apparent digestible energy coefficient (ADEC) was not affected by the treatments [$F(2, 10) = 1.65$, $P = 0.24$; Figure 6a]. The metabolizable energy coefficient (MEC) was reduced by resin [$F(2, 10) = 5.21$, $P = 0.028$; Figure 6b]. MEC was lower in woodrats fed the resin-treated diet than in control 1 [$F(1, 5) = 10.17$, $P = 0.024$]. There was a similar trend between resin and control 2 [$F(1, 5) = 4.21$, $P = 0.095$; Figure 5b]. Urinary energy excretion as a percentage of apparent digestible energy was higher in woodrats fed resin than in control diets [$F(2, 10) = 20.26$, $P < 0.001$, control 1 vs. resin: $F(1, 5) = 23.15$, $P = 0.005$; control 2 vs. resin: $F(1, 5) = 23.89$, $P = 0.005$]. There were no

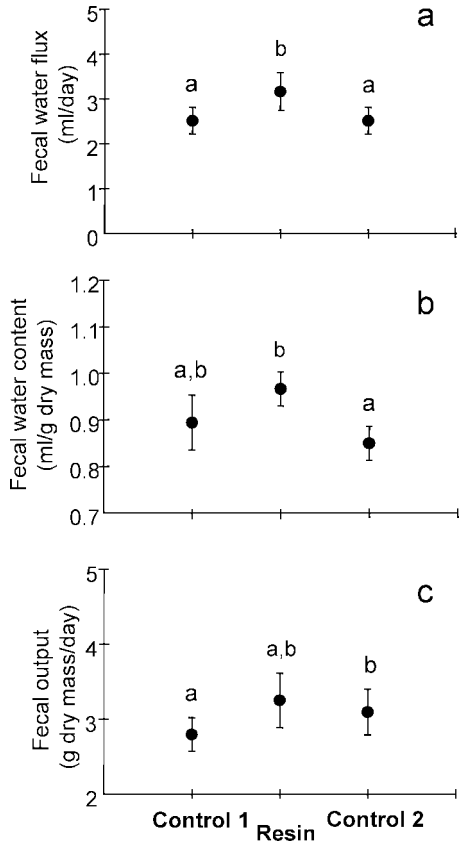

FIG. 3. Variation of fecal water flux (a), fecal water content (b), and fecal dry mass output (c) of desert woodrats consuming control versus resin diets for experiment 1. Values are expressed as mean plus or minus one standard error. Means with the same lowercase letter indicate no significant difference. Differential contrasts were used to test for difference between continuous treatments in experiment 1 and special contrasts to test for differences between control 1 and control 2.

differences between contro1 1 and control 2 with respect to urinary energy excretion [$F(1, 5) = 1.61$, $P = 0.25$; Figure 7].

DISCUSSION

The addition of creosote resin to the diet affected both the minimum water required and the energy available for woodrats. To our knowledge, this is

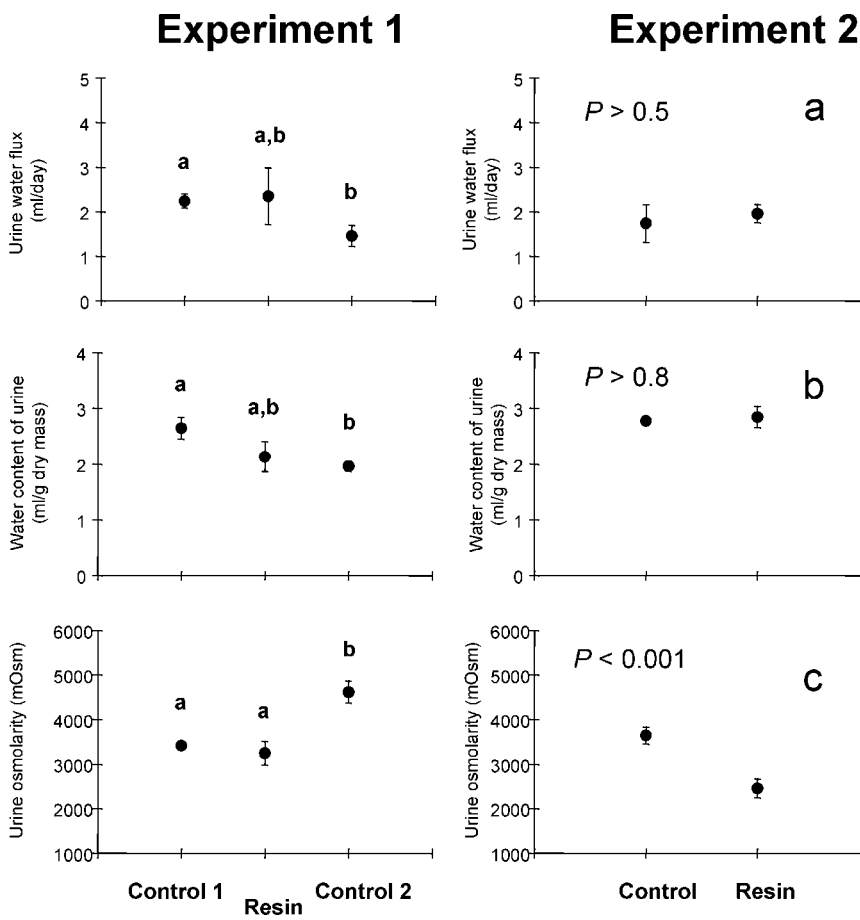

FIG. 4. Variation of urinary water flux (a), water content (b), and osmolarity of the urine (c) of desert woodrats fed control or resin diets for experiments 1 and 2. Values are expressed as mean plus or minus one standard error. Means with the same lowercase letter indicate no significant difference. Differential contrasts were used to test for difference between continuous treatments in experiment 1 and special contrasts to test for differences between control 1 and control 2. For experiment 2, P values represent the overall significance for the repeated measures ANOVA with one grouping factor (the sequence in which the diet was offered, either control-resin or resin-control).

the first experimental evidence on the effects of plant secondary metabolites on minimum water requirement of a herbivorous mammal. Our results support the hypothesis that the minimum water required for woodrats to maintain body mass increased with the ingestion of creosote resin. Also, we confirmed the prediction that

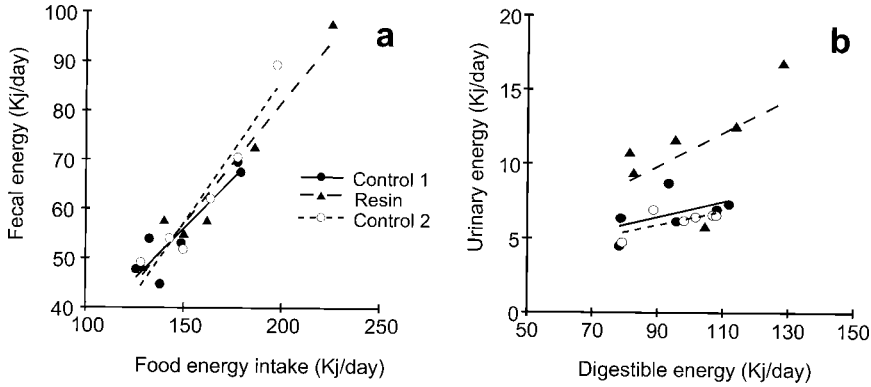

FIG. 5. Fecal energy output vs. food energy intake (a) and urinary energy excretion vs. digestible energy (b).

metabolizability of dietary energy was lower for woodrats fed resin diets compared to control diets.

Effect of Resin on Water Intake. The addition of resin to diet increased water intake and the minimum water requirement. The minimum water requirement for desert woodrats on control diets was 5.5 ml/d compared to 7 ml/d when resin was added (Figure 2; experiment 2). In comparison, *ad libitum* drinking rates are 12 ml/d for control diets vs. 15 ml/d for diets with 1–3% resin, and 25 ml/d for 5% resin diets (Mangione et al., 2000). Clearly, when woodrats are given free access to water, they drink in considerable excess of the minimum they need to match minimum water losses. When resin was added to the diet, the minimum requirement increased by 18–30% (1–1.7 ml/d above 5.5 ml/d). This increase (25%) is comparable to that observed for *ad libitum* intake of water by woodrats fed a 3% resin diet. In addition, ingestion of resin reduces estimates of metabolic water by reducing the amount of food available to be metabolized.

Effect of Resin on Avenues of Water Loss. On control diets, woodrats lost similar amounts of water (~2.5 ml/d) through each of the three possible avenues (urine, fecal, and evaporative). Resin appeared to increase minimum water loss rate mainly through an effect on fecal water loss. Fecal water loss rate was 0.7 ml/d higher in woodrats eating resin than in controls, and accounts for 70% of the difference in required water between control and resin diets. The increase in fecal water loss in woodrats eating resin was due to increases in both fecal water content and fecal dry matter excreted.

An increase in fecal water content may occur if fewer osmolytes are absorbed from the intestinal lumen causing retention of water by osmosis or by increasing the secretory rate of water. A component of creosote resin, NDGA, inhibits

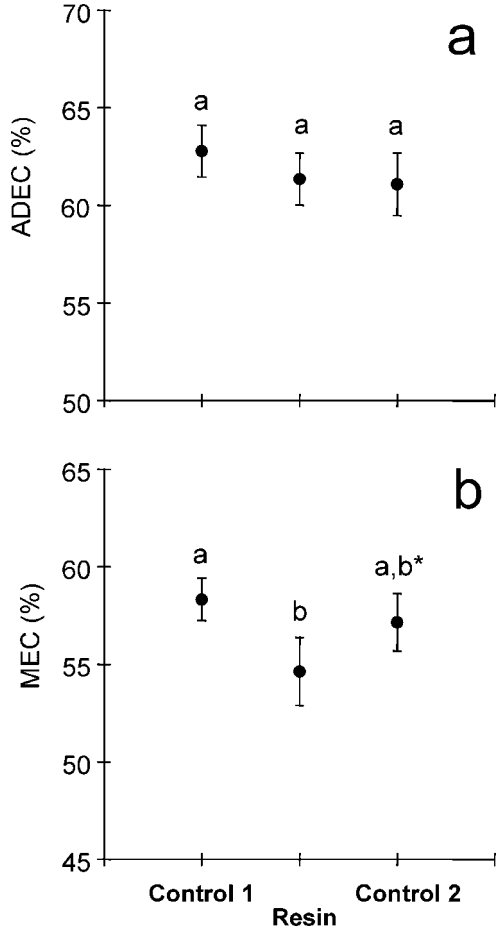

FIG. 6. Apparent digestible energy (a) and metabolizable energy coefficient (b) with treatments. Different letters indicate means are significantly different ($P < 0.05$). The asterisk indicates that the difference between resin and control 2 was significantly different ($P < 0.1$).

Na^+ K^+ ATPase of intestinal mucosa homogenates in rats (Kellett et al., 1993). The disruption of Na^+ transport may alter the mechanisms of solute-coupled water absorption in the large intestine. Thus, it is plausible that resin had a direct effect on osmolyte absorption. References regarding the effects of plant secondary compounds on Na^+ balance, retention, or excretion are abundant (Freeland et al., 1985; Navarro et al., 1994; Johnson et al., 1999; Dearing et al., 2001, and references

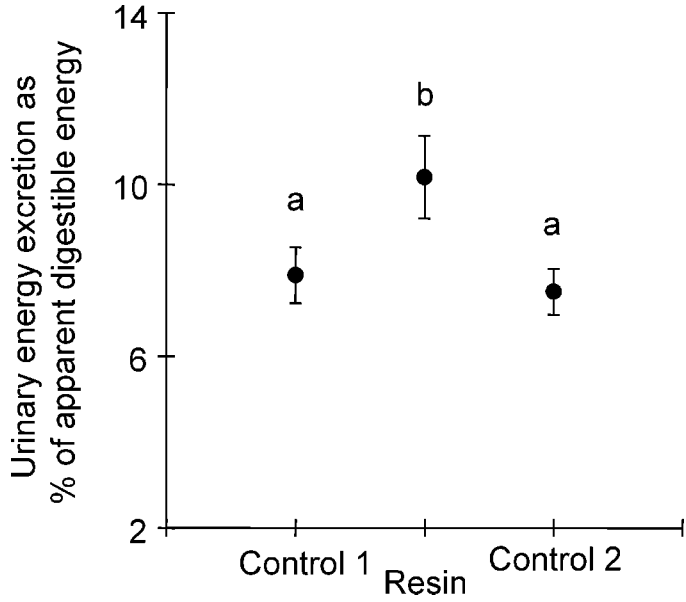

FIG. 7. Urinary energy excretion as a percentage of apparent digestible energy. Means with different letters are significantly different ($P < 0.05$).

therein). Most of these studies focused on the natruretic and diuretic effects of these compounds on animals, mainly laboratory rats (Galati et al., 1996). Dearing et al. (2001) also pointed out that the effect of secondary compounds on the water balance of wild herbivores has received little attention, but could be of tremendous importance to the fitness of the animal.

Increases in production of fecal dry matter could occur if resin ingestion reduced digestive efficiency (more feces produced per unit food consumed) or if larger quantities of resin-containing food were consumed. The first explanation seems unlikely, as Meyer and Karasov (1989) showed that resin had no significant effect on dry matter digestion, an observation we confirmed. The second explanation is not strongly supported by our data because food intake did not increase resin-treated diet (Figure 2).

Resin had relatively little effect on urinary or evaporative water loss. Although evaporative water loss differed significantly among treatments in experiment 1, it was not consistently higher in woodrats eating resin, but increased progressively with time. We do not know whether this reflects chronological changes in the woodrats themselves, which were all adults. It is possible that environmental conditions, e.g., relative humidity, which were thought to have been held relatively

constant, may have varied somewhat as the animal room was not an environmental chamber, but a standard animal facility.

The increase in minimum water requirement was not matched by a significant increase in urinary water loss. This was somewhat surprising, as NDGA has been reported to have a diuretic effect on humans (Timmermann, 1977). In addition to NDGA, many other PSMs have diuretic properties (Dearing et al., 2001). Moreover, 3% NDGA fed to lab rats produced cysts in their kidneys (Grice et al., 1968). This effect could decrease the kidney's capacity to minimize urinary water loss. However, neither the water content of the urine, nor the urinary water flux, was significantly affected by the resin (Figure 4). Curiously, urine osmotic pressure was lower in woodrats eating resin diets (Figure 4). Although these results imply a diuretic effect of resin, a simple comparison of osmolarity may be confounded by the different osmolytes excreted on the control vs. resin diets. Evidence of a substantial difference in osmolytes is indicated by the higher energy density and excretion of glucuronic acid metabolites in urine of animals consuming resin diets (Mangione et al., 2001). The results imply a change in urinary osmolytes; however, more research on the chemical composition of the osmolytes is necessary to explain these findings.

Ecological Implications of Increased Minimum Water Requirement. Even a small increase in the minimum water requirement of an animal, particularly a desert one, could result in myriad ecological consequences. The case documented by Karasov (1989) of woodrats feeding exclusively on creosote during the winter exemplifies the possible ramifications. In this case, the suitability of creosote as the only source of preformed water was marginal for desert woodrats, independent of the effect of creosote resin. The water content of the majority of individual creosote bushes measured by Karasov (1989) was not sufficient to meet the water requirements of woodrats on resin-free diets. The ingestion of creosote resin magnifies the problem: because the water requirement is greater when woodrats consume resin, even fewer creosote bushes than that estimated by Karasov (1989) will contain enough water to satisfy their water requirements. To meet water requirements, woodrats must be extremely selective in their choice of creosote bushes. Such dietary selectivity may necessitate that they travel longer distances between bushes with adequate water contents, thereby increasing the risk of predation. Furthermore, water requirements may be additionally enhanced via increases in evaporative water losses caused by increased movement associated with selective foraging. The difficulties associated with maintaining water balance while consuming creosote could be further exacerbated if resin concentration and water content were positively correlated. The water content of branches within a creosote bush varies significantly, with the top parts of the bush having greater water contents than the lower parts (Karasov, 1989). Resin content is higher in young leaves than in mature leaves within a bush (Meyer and Karasov, 1989). Thus, by

feeding on mature leaves on the upper portions of bushes, woodrats may maximize water intake and minimize resin intake.

Other mammals that feed on creosote may also be foraging selectively to optimize the water to resin ratio. According to Ernest (1994), jackrabbits (*Lepus californicus*) prefer to repeatedly forage from mature branches rather than current-year-growth branches, but water content was not measured in that study. Another example is *Ctenomys mendocinus*, a fossorial rodent from the Central Monte in Argentina, that forages on creosote bush (*Larrea cuneifolia*). There is no evidence of preference by *C. mendocinus* for water or resin content of *Larrea cuneifolia* branches (A. M. Mangione, 1990, 2002, personal observation). The interplay of resin and water on the water balance of desert herbivores and subsequent ecological consequences deserves further consideration.

Effect of Resin on Energy Availability. Our prediction that the metabolizability of dietary energy would be lower in animals fed resin diets compared to control diets was confirmed. Urinary energy loss was 40% higher in woodrats fed resin-treated diets compared to control diets, and the percentage of digestible energy excreted in woodrat urine was 24% higher on resin diets than control diets (Figure 5b). Woodrats had 4.5 and 6.3% lower MEC when fed resin-treated diets than control diets (Figure 6b). Resin had no effect on the percentage of ingested energy that was apparently digested (Figure 5a).

We have confirmed by two different methods (ratios and ANCOVA) that ingestion of creosote resin significantly increases urinary energy losses in woodrats fed resin-treated diets compared to control diets. How do these values compare to other values given in the literature, and is the magnitude of the energy drain biologically relevant? Jakubas et al. (1993a) and Guglielmo et al. (1996) suggested that herbivorous mammals have lower costs of detoxification via the glucuronic acid pathway than birds. According to these authors, the energy excreted in the form of glucuronic acid by herbivorous mammals represents 0.6–1.2% of the metabolizable energy intake (MEI = $\mathrm{Ge}_i Q_i$*MEC), and this percentage is considerably greater in birds eating natural forages (2–25 times). We estimated production of glucuronic acid in this study, from the previously established relationship between glucuronide excretion and maximum resin intake for Mojave woodrats (Mangione et al., 2001) [Glucuronide (mg/d) $= -0.41 + 0.457 *$ resin intake (mg/d)]. We incorporated the values of resin intake of this study (estimated from the feeding rate) into the equation above. The total energy associated with the excretion of glucuronic acid is the product of glucuronic acid excretion and its heat of combustion (13.5 kJ/g; Guglielmo et al., 1996). The energy lost as glucuronic acid was expressed as percentage of MEI. Our calculations reveal that energy in glucuronic acid was 1.9% of MEI, which is 1.6× greater than the highest values reported for mammals and similar to some of the values reported for birds (Jakubas et al., 1993a; Guglielmo et al., 1996). Total urinary energy excreted,

which includes glucuronic acid as well as other detoxification metabolites, was 12.4% of MEI in woodrats fed resin-treated diets compared to 7.3% for woodrats on control diets. This implies that almost half of the urinary energy lost as a consequence of creosote resin ingestion, may be the result of energy lost in the form of glucuronic acid, glycine, and sulfate conjugates, with glucuronic acid comprising 75% of the total moles of conjugates excreted in urine (Mangione et al., 2001).

The hypothesis that there is a lower cost of detoxification in mammals than in birds requires further evaluation. In this study, we fed woodrats naturally occurring allelochemicals (creosote resin) and the cost of detoxification as a percentage of MEI was similar to some of the values given for birds (Guglielmo et al., 1996). Moreover, the percentage of digestible energy excreted in the urine of woodrats fed resin-treated diet (10.2%) was similar to reported values for rodents and lagomorphs fed forage containing PSMs (green wheat and forbs; Robbins, 1993). The hypothesis that birds have greater detoxification costs can be properly tested only by feeding birds and mammals diets containing similar secondary metabolites.

In summary, plant secondary metabolites are thought to deter herbivores by toxicity and by reduction of digestibility of matter and/or nitrogen. This study quantified the two other costs: energy and water. Even if a herbivore has mechanisms to cope with the toxicity of PSMs, it may still confront the negative effects that PSMs exert on water and energy balance. These negative effects of PSMs should be factored into the numerous potential detrimental effects of secondary compounds on herbivore performance.

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ENDOGENOUS FREE FATTY ACIDS REPEL AND ATTRACT COLLEMBOLA

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Abstract—We used video recording of the movement pattern of *Protaphorura armata* (Collembola) to test whether its avoidance of the odor of dead conspecifics extends to related species. *P. armata* was repelled by the odor of dead individuals of *Onychiurus scotarius* and *Onychiurus circulans*, but not by live individuals. Free palmitic, oleic, and linoleic acids were present in extracts of the three repellent species, but only free palmitic acid was detected in extracts of a fourth nonrepellent species, *Folsomia candida*. Synthetic palmitic acid was attractive to *P. armata*, linoleic acid was repellent, and oleic acid gave no response. *O. scotarius* and *O. circulans* also contained 2,3-dimethoxy-pyrido[2,3-b]pyrazine, known as a defense substance. We discuss the role of free fatty acids in predator avoidance, conspecifics attraction, and food recognition in *P. armata*.

Key Words—Collembola, *Protaphorura armata*, movement, attraction, repellence, alarm, fatty acid, pyridopyrazine, defense.

INTRODUCTION

Prey show a variety of adaptations to reduce the risk of predation, such as defense, e.g., spines, armor, and toxins, and avoidance behavior, e.g., hiding, fleeing, and delayed hatching. The predation risk can be assessed by visual and mechanical cues, but is also commonly estimated by chemical cues (Kats and Dill, 1998). The origin of the chemical cues may be the predator, or alarmed, injured, or dead conspecifics (Dicke and Grostal, 2001). All prey that share habitat and potential predators would benefit from having the capability to detect alarm substances from conspecifics, closely related species, and phylogenetically distant species (Mathis and Smith, 1993).

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The release of chemicals by threatened, caught, or dead individuals to warn conspecifics or heterospecifics can be explained in several ways. First, chemicals may be released to alarm conspecifics (alarm pheromone), which assumes kinship relations to the receiver (Maynard Smith, 1965). Several alarm pheromones have been identified (Bowers et al., 1972; Hölldobler and Wilson, 1990; Hunt et al., 2003) as predominately short-chained volatile compounds (Hölldobler and Wilson, 1990). Second, chemicals may be released as a predator defense. Defense substances are present in many organisms and have a wide biochemical diversity (Berenbaum, 1995). There are also compounds with a dual function as alarm substance and predator repellent (Blum, 1996, and references therein). Third, the active compound may be a metabolic byproduct, e.g., in the hemolymph. Free fatty acids from crushed cockroaches repel conspecifics (Rollo et al., 1994), a phenomenon that can be compared with the necrophoric behavior in ants and bees. The authors suggested that fatty acids may be a ubiquitous death-recognition signal.

Little is known about chemical ecology of soils, although the darkness of the medium should favor the development of chemical cues rather than visual cues. This is illustrated by the complex chemosensory repertoire of *Caenorabditis elegans*, a soil-living nematode (Troemel, 1999). Injury-released substances that repel conspecifics are known from several Collembola families (Usher and Balogun, 1966; Purrington et al., 1991; Messer et al., 1999), and the alarm substance has been identified for one species (Messer et al., 1999). *Protaphorura armata* (Collembola) is repelled by the odor of dead conspecifics, but ignores dead individuals of a phylogenetically distant relative, *Folsomia candida* (Nilsson and Bengtsson, 2004). To reveal the pattern of phylogenetic relatedness of the recognition of the death odor, a series of experiments were designed to test whether the repellence of the odor of dead conspecifics extends to closely related species of *P. armata* and, if so, identify the substances responsible for the repellence.

METHODS AND MATERIALS

Organisms. Collembola of the species *Folsomia candida* (Willem) came from an in-house culture (originally thousands of founder individuals from DMU, Silkeborg, Denmark). *P. armata* (Tullberg) came from an in-house culture that had been supplied in periods with thousands of individuals from the same deciduous forest soil near Lund (55°43′N 013°12′E). *Onychiurus circulans* (Gisin) was from soil beneath a compost of garden litter near Landskrona (55°52′N 012°50′E) and *Onychiurus scotarius* (Gisin) from a compost in Ramlösa (56°03′N 012°42′E). The latter two species were used in the experiments without prior culturing. The Collembola were kept enclosed in darkness at 12°C in Petri dishes (90 mm in diam) with a bottom of moistened plaster of paris and activated charcoal (9:1). They were continuously fed with the fungus *Verticillium bulbillosum* (W. Gams and Malla).

Two-Phase Extraction. Sixty *P. armata*, divided into four Eppendorf vials, were crushed by a metal peg and extracted with a mixture of chloroform, methanol, and phosphate buffer (50 mM K_2HPO_4, pH 7.4) in the proportions 1:2:0.8 (v/v). The solid residue and solvent mixture were transferred into 7-ml test tubes of glass with screw caps to minimize the risk of solvent leaking. The Eppendorf vials were rinsed with a new portion of the solvent mixture, and the rinse was added to the test tubes, resulting in a final volume of 2.5 ml. The test tubes were vortexed for 1 min, left for 2 hr at room temperature, and then centrifuged (Hermle Z 230A) for 5 min at 2500 rpm. The supernatant was transferred into a clean test tube. The residue (remaining animals) was washed once with 1.0-ml solvent mixture. The supernatants were combined. The phases were separated by adding 1.0-ml chloroform and 1.0-ml phosphate buffer to get chloroform, methanol, and phosphate buffer in the proportions 2:2:1.8. The extract was left overnight for phase separation. The samples were then centrifuged for 10 min at 2500 rpm; the upper aqueous phase was transferred into a new test tube, and the bottom organic phase was left in the old test tube. Both phases were evaporated to dryness by vacuum centrifugation at room temperature (Savant Speedvac with VaporNet®AES 1000). The samples were stored in a freezer at −20°C. Before use, impurities were removed from the test tubes by heating for 12 hr at 400°C. For the bioassays, 200 μl of cyclohexane was added to the organic extract. The aqueous extract was not totally evaporated and used without dilution.

Single-Phase Extraction. Cyclohexane was used for extraction because previous work with other Collembola species indicated that a repellent might be nonpolar (Purrington et al., 1991). Four hundred *O. circulans* were put into an Eppendorf vial and crushed by a metal peg. The crushed animals were vortexed with 1 ml of cyclohexane for 1 min, and the mixture was left for 2 hr at room temperature. The contents were transferred to a 7-ml glass test tube with screw cap and centrifuged for 5 min at 2500 rpm at room temperature. The supernatant was transferred to a clean glass vial. The same procedure was used to extract 400 *O. scotarius* and 200 *P. armata*. The supernatants were stored in a freezer at −20°C.

Bioassay. Observations of the movement pattern were made on Petri dishes (90 mm in diam) half-filled with clay (Skromberga clay Hb20; 45% quartz, 5% chlorite, 50% illite), dyed to black by adding Fe_3O_4 (8:1). The clay surface was replaced between each set of observations so that odor from a previous individual should not affect the movement pattern of the next one. The Petri dish was divided into two equally large areas, one of them, the test area, was prepared as described below. The other area was the control area.

Different treatment and movement observations were made in a temperature-controlled room (20 ± 0.5°C). Video recordings were made in red light from four light sources (red darkroom lamp, 230V Philips). One individual at a time was placed into the Petri dish and tracked for 15 min. The dish was covered with a

glass lid during recording to retain moisture and avoid disturbance. Each treatment was replicated 10 times. The image of the Petri dish was captured by a monochrome video camera (Cohu 4710) and digitized by a framegrabber (VIGA+) connected to a personal computer. EthoVision®(Noldus Information Technology, Wageningen, the Netherlands), which provides both object detection and data analysis, was used for image processing. Detection of animals was based on a grey scaling. One image per second was processed and resulted in a time series of X, Y-coordinates indicating the position of the animal.

First, we tested whether *P. armata* was repelled by dead individuals of two other species of the *Onychiuridae* family, *O. scotarius* and *O. circulans*. Dead individuals of *P. armata* and *F. candida* were not tested since the reaction of *P. armata* to dead conspecifics and dead individuals of *F. candida* was known (Nilsson and Bengtsson, 2004). Three individuals of either species were killed in the test area and the content of the bodies smeared on the surface with a brush. As a comparison, 10 live individuals of either species were released into the test area for 12 hr, removed, and replaced by one individual of *P. armata*, which was observed for 15 min.

Second, we observed the movement of *P. armata* in the Petri dish when the test area was treated with whole-body extracts of *Onychiuridae* species. We used extracts from the two methods described above, two-phase and single-phase extraction. Extracts (10 μl/replicate) were added by brush to the test area of the clay surface and the same amount of pure solvent (either water or cyclohexane) was added to the control area. The extract and solvent were left for 10 min to evaporate before *P. armata* was introduced.

Third, the movement of *P. armata* was observed in presence of synthetic fatty acids ($\geq$99% pure, Larodan Fine Chemicals AB, Limhamn, Sweden). Aliquots of 10-μl cyclohexane containing one fatty acid, 45 μg of palmitic (hexadecanoic), 55 μg of stearic (octadecanoic), 38 μg of oleic (9-octadecenoic), 55 μg of linoleic acid (9,12-octadecadienoic), or 55 μg of linolenic acid (9,12,15-octadecatrienoic) were added to the test area. Linoleic acid was also tested at two additional concentrations, 5.5 and 0.6 μg. The same amount of pure cyclohexane was added to the control area. Cyclohexane was left for 10 min to evaporate before *P. armata* was introduced.

Chemical Analysis. Extracts for gas chromatography – mass spectrometry (GC-MS) analysis were prepared as described above for extraction with single-phase solvent, but with 7 mg of *P. armata*, *O. scotarius*, *O. circulans*, or *F. candida* in 100 μl of cyclohexane instead of 400 animals in 1-ml cyclohexane. Blanks containing cyclohexane were also prepared in the same way. The extracts were stored in a freezer at -20°C before use.

Chemical analyses were performed on a combined gas chromatograph and mass spectrometer (GC-MS): Hewlett-Packard (HP) 6890 GC and an HP 5973 MS. All samples were automatically injected with an HP 7683 pulsed splitless injector

(175 kPa for 0.5 min). GC was equipped with a 30 m × 0.25 mm fused silica column coated with HP-5 (5% phenyl and 95% methyl siloxane, df = 0.25 μm: Agilent Technologies, Palo Alto, CA, USA). Temperature programming was 50°C for 2 min, 10°C/min to 300°C, and isothermal at 300°C for 10 min. The injector temperature was 275°C, and the transfer line was programmed at 225°C for 20 min and 10°C/min to 275°C, and then kept isothermal. Helium was used as carrier gas at 30 cm s^{-1}, and the electron impact (EI mode) mass spectra were obtained at 70 eV. Compounds were identified by their GC retentions times and mass spectra, and compared with authentic samples of synthetic references. Mass spectra were also compared to commercially available MS libraries (NBS and Wiley).

Statistical Analysis. EthoVision® was used to extract the time spent in each of the two areas of the Petri dish by each individual. The time spent in the control and test area in the Petri dish was compared with a paired *t* test using SPSS (SPSS Inc.) with each individual treated as a replicate. The individuals were considered repelled or attracted to the cues tested when the time spent in the control area was significantly longer or shorter than in the test area.

RESULTS

P. armata was repelled by the residues of dead individuals of both *O. circulans* ($t = -3.06$, $P = 0.02$) and *O. scotarius* ($t = -3.64$, $P = 0.01$), but insensitive to traces of live individuals of them (Figure 1). The aqueous phase of the two-

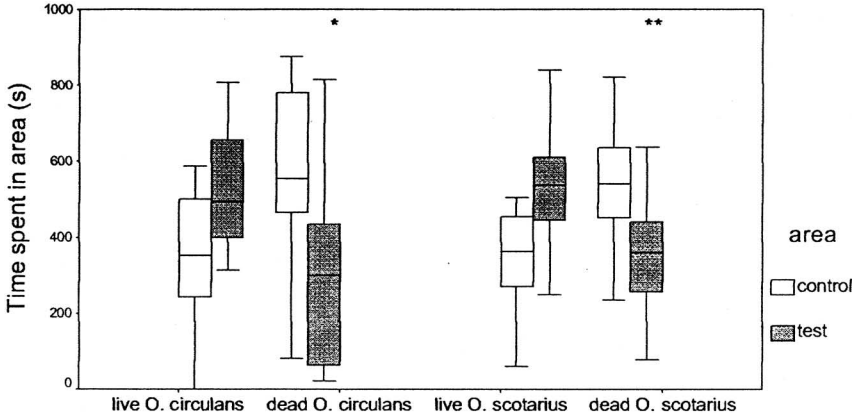

FIG. 1. Time spent by *P. armata* during recording of movement for 15 min in control and treated areas. The treated (test) area was conditioned with live *O. circulans*, dead *O. circulans*, live *O. scotarius*, or dead *O. scotarius*. The plot gives the 10th, 25th, 50th (median), 75th, and 90th percentiles of the variable. Significance levels are illustreated by asterisks, $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$.

TABLE 1. TIME SPENT BY *P. armata* IN CONTROL AND TREATED AREAS DURING RECORDING FOR 15 MIN

Species (number of animals used)	Extraction method	Time spent in the area(s)(±SE)[a]	
		Control area[b]	Test area[c]
P. armata (60)	Two phase, aqueous phase	510 ± 47	388 ± 47
P. armata (60)	Two phase, organic phase	470 ± 26	428 ± 25
P. armata (200)	Single phase, cyclohexane	370 ± 60	530 ± 60
O. circulans (400)	Single phase, cyclohexane	468 ± 47	430 ± 46
O. scotarius (400)	Single phase, cyclohexane	749 ± 45	144 ± 44***

[a] Significance level is illustrated by asterisks, *** $P \leq 0.001$.
[b] Treated with pure solvent.
[c] Extracts of *P. armata*, *O. circulans*, or *O. scotarius* were applied to the treated (test) area before the movement was recorded.

phase extract was weakly repellent ($t = 1.30$, $P = 0.21$; Table 1), but the organic phase had no activity. The cyclohexane extract of 400 *O. scotarius* was repellent to *P. armata* ($t = 6.79$, $P < 0.001$), whereas that of 400 *O. circulans* was not (Table 1). *P. armata* was weakly attracted by the cyclohexane extract of 200 conspecifics ($t = -1.33$, $P = 0.21$; Table 1).

The gas chromatogram of the repellent extract of *O. scotarius* had three peaks that were not found in the controls. The peaks were identified as (1) 2,3-dimethoxypyrido[2,3-b]pyrazine, (2) palmitic, and (3) oleic and linoleic acid (Figure 2) by their GC retention times and mass spectra, and compared with authentic samples

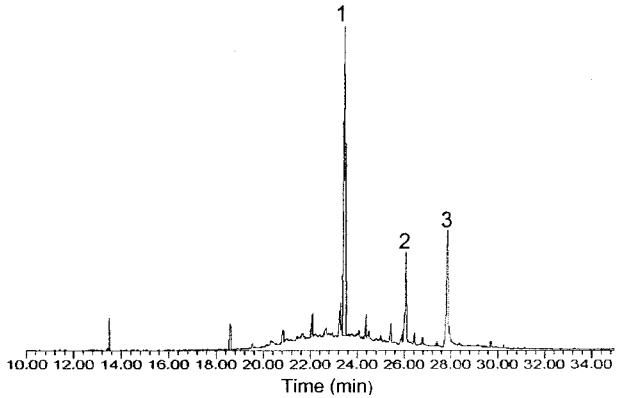

FIG. 2. Gas chromatogram of repellent cyclohexane extract (whole-body extraction with pure solvent) of *O. scotarius*. The peaks were identified as (1) 2,3-dimethoxy-pyrido[2,3-b]pyrazine, (2) palmitic, (3) oleic and linoleic acids by their retention times and mass spectra.

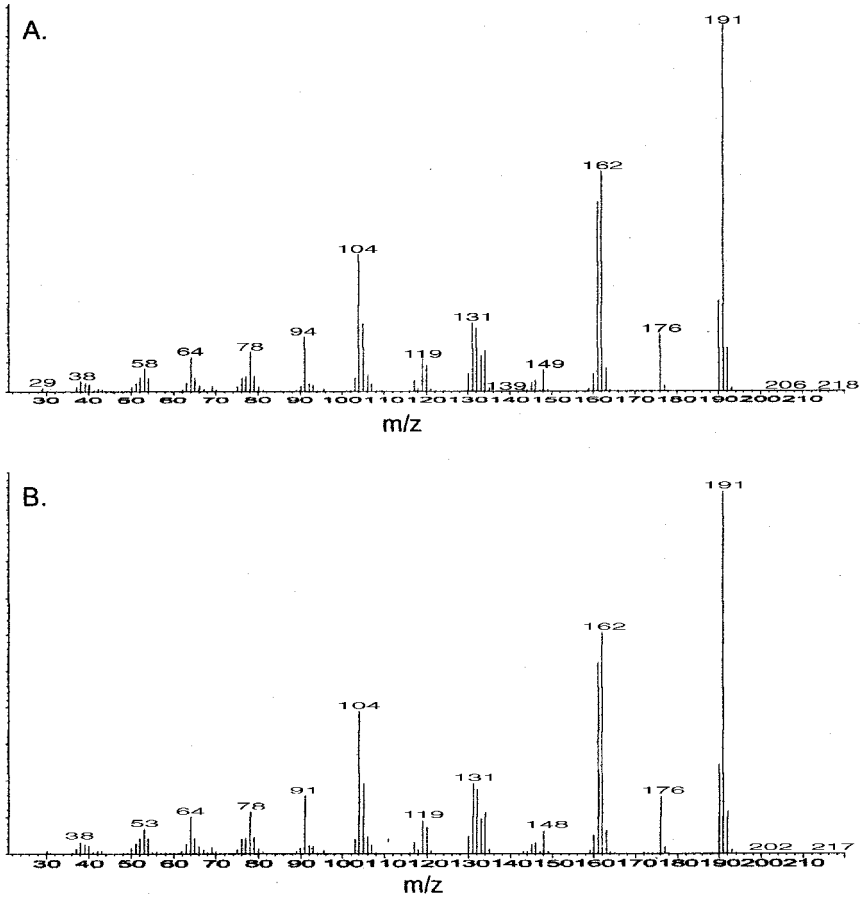

FIG. 3. Mass spectra of (A) 2,3-dimethoxy-pyrido[2,3-b]pyrazine in extract of *O. scotarius* and (B) 2,3-dimethoxy-pyrido[2,3-b]pyrazine (reference substance).

of synthetic references (Figure 3). 2,3-Dimethoxy-pyrido[2,3-b]pyrazine was only found in *O. scotarius* and *O. circulans* and none of the substances except palmitic acid were found in *F. candida*. The highest concentrations of all of the substances were found in *O. scotarius* (Table 2).

We used the observed concentrations (Table 2) to calculate an approximate amount of fatty acids found in 500 animals (*O. scotarius*) and added that amount of synthetic fatty acids to the test area. *P. armata* was only repelled by linoleic acid ($t = 3.52$, $P = 0.01$) (Figure 4). Palmitic acid was attractive ($t = -3.13$, $P = 0.01$) and oleic acid gave no response (Figure 4). To rule out a general response to free fatty acids as an explanation for those observations, we also tested stearic

TABLE 2. CONCENTRATION OF SUBSTANCES IDENTIFIED IN CYCLOHEXANE EXTRACTS OF FOUR SPECIES OF COLLEMBOLLA

Species	Substance			
	2,3-Dimethoxy-pyrido [2,3-b]pyrazine (mg/g[a])	Palmitic acid (mg/g[a])	Oleic acid (mg/g[a])	Linoleic acid (mg/g[a])
P. armata	nd[b]	0.03	0.02	0.05
O. scotarius	0.9	0.5	0.4	0.6
O. circulans	0.8	0.1	0.3	0.06
F. candida	nd	0.03	nd	nd

[a] mg/g wet weight.
[b] nd = not detectable.

and linolenic acid although they were not detected in the extracts. *P. armata* did not respond to them (Figure 4). Linoleic acid was repellent at a dose corresponding to 50 animals ($t = 3.17$, $P = 0.01$), but the effect of a dose corresponding to 5 animals was insignificant (Figure 5).

DISCUSSION

Linoleic acid was the strongest *P. armata* death-repellent candidate from among the compounds tested. It was present in the cyclohexane extract of all three *Onychiuridae* species. In addition, linoleic acid was the only repellent from among the pure substances. Pure oleic acid, which was also found in all *Onychiuridae*,

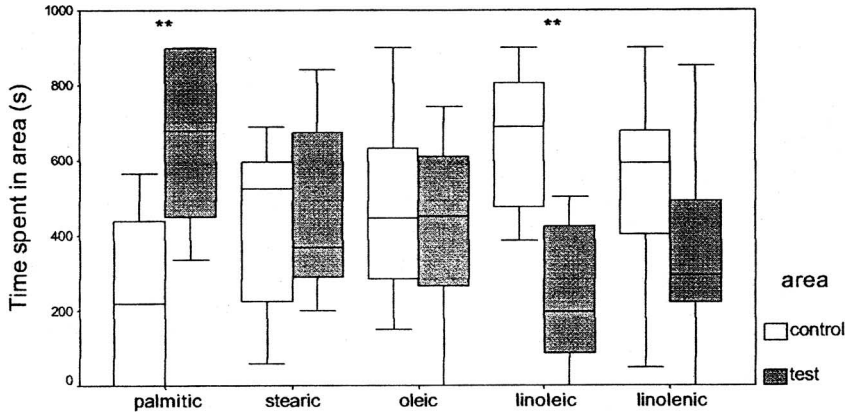

FIG. 4. Time spent by *P. armata* during recording of movements for 15 min in control and treated areas. The treated (test) area was conditioned with palmitic, stearic, oleic, linoleic, or linolenic acid. The plot gives the 10th, 25th, 50th (median), 75th, and 90th percentiles of the variable. Significance levels are illustrated by asterisks, $^{**}P \leq 0.01$.

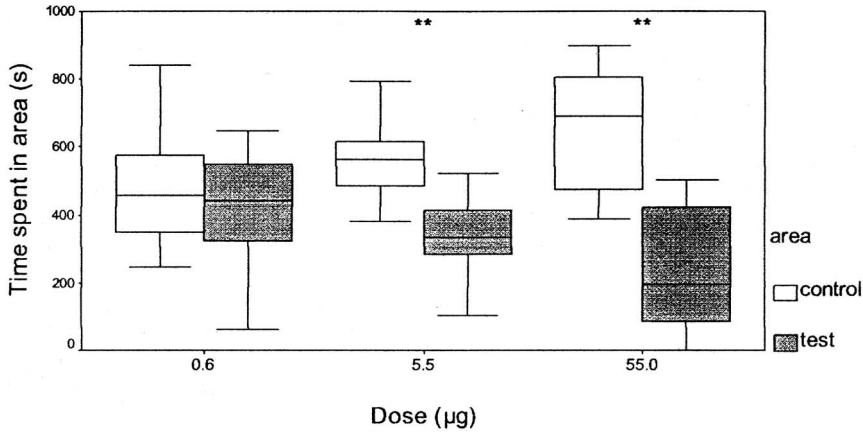

FIG. 5. Time spent by *P. armata* in control and treated areas during recording of movement for 15 min. The treated (test) area was conditioned with linoleic acid at doses corresponding to the amount found in extracts of 5 (0.6 μg), 50 (5.5 μg), and 500 (55.0 μg) individuals. The plot gives the 10th, 25th, 50th (median), 75th, and 90th percentiles of the variable. Significance levels are illustrated by asterisks, $^{**}P \leq 0.01$.

but not in *F. candida*, can be removed from the candidate list since it was not repellent to *P. armata*. 2,3-Dimethoxy-pyrido[2,3-b]pyrazine was removed from the list of individual repellents because it was not detected in *P. armata* and because the cyclohexane extract of *O. circulans* was inactive (Table 1), although 2,3-dimethoxy-pyrido[2,3-b]pyrazine was found at the same high concentration in both *O. scotarius* and *O. circulans*.

A prey can respond to both direct (originating from the predator) and indirect (originating from the prey) predator cues. The latter response is thought to be an adaptation to nonspecialist predators or those that are not inherently recognized by the prey (Grostal and Dicke, 1999). Many springtails are preyed upon by a variety of generalist predators (Foster, 1970; Ernsting and Joosse, 1974; Johnson and Wellington, 1980; Schlegel and Bauer, 1994), and the recognition of each of them would require an elaborate perception system. The odor of injured conspecifics or heterospecifics may precede the odor of a predator (e.g., from predator feces) and make its detection earlier (Grostal and Dicke, 2000).

The observation that linoleic acid in dead conspecifics and heterospecifics of the *Onychiuridae* family was repellent to *P. armata* gives support to the suggestion by Rollo et al. (1994) on the importance of free fatty acids as general death-recognition cues to avoid areas with high risk of predation, parasites, or diseases. Ants, aphids, beetles, and cockroaches are repelled by linoleic and/or oleic acid (ants: Howard et al., 1982; aphids: Greenway et al., 1978; beetles: Nijholt, 1980; cockroaches: Rollo et al., 1994). The reason why repellence to cyclohexane extracts

of *P. armata* and *O. circulans* failed to appear may be the low amount of linoleic acid due to smaller and fewer individuals (*P. armata*) and/or lower body concentration than in *O. scotarius*. Dead *F. candida* may not be repellent to *P. armata* because it lacks free linoleic acid (Table 2). It is possible that *F. candida*, which can use a furca to escape from predators, does not share predators with *P. armata*. If so, *P. armata* would have no benefits from recognizing dead individuals of *F. candida*. Both phospholipids and neutral lipids of *F. candida* were found to contain linoleic acid, but the amount of free linoleic acid was not analyzed (Holmstrup et al., 2002). The neutral lipid fraction was dominated by oleic and palmitic acid, but we found only free palmitic acid. In contrast, Fitters et al. (1999) found that the phospholipid, neutral lipid, and free fatty acid fraction of nematodes had the same composition of the most abundant fatty acids.

The source of the free fatty acids in *P. armata* is unknown. In insects, in general, fatty acids are absorbed from food, converted into diglycerides in the midgut, and transported into the hemolymph by lipophorin. Once in the target tissue, the diglycerides are reconverted into free fatty acids (Canavoso et al., 2001). Fatty acids are either stored in the fat body as triglycerides and used as an energy source, or used as structural components for cell membranes (Canavoso et al., 2001). Fatty acids also serve as precursors for many insect pheromones (Tillman et al., 1999); however, pheromones have not been identified in *P. armata*.

If linoleic acid was this general death-recognition cue, one would expect it to be present in prey animals, but not in their food. Collembola feed on fungal hyphae (Visser et al., 1987; Draheim and Larink, 1995), which contain palmitic, stearic, oleic, and also linoleic acid from phospholipids and triglycerides (Müller et al., 1994; Stahl and Klug, 1996; Chen et al., 2001). The amount of free fatty acids is rarely determined, but seems to be less than the amount of triglycerides, with the same relative composition of fatty acids (Beilby and Kidby, 1980). Therefore, linoleic acid is likely to be present as free fatty acid in fungi, but the repellent effect may be moderated by other cues, e.g., free palmitic acid. Although the same fatty acids dominate in all fungi, the relative amounts vary from one species to another (Stahl and Klug, 1996). It is possible that *P. armata*, which is known to prefer some species of fungi to others (Bengtsson et al., 1985; Shaw, 1988; Chen et al., 1995), uses the relative abundance of attracting and repelling fatty acid in their food species selection. It is also known that Collembola prefer young hyphae to old (Leonard, 1984) and that the fatty acid composition of fungi changes over time (Stahl and Klug, 1996).

The greatest amount of synthetic fatty acids used in the bioassay corresponded to 15 dead individuals/cm^2. Collembola can be abundant in soil (up to 60,000 individuals/m^2; Hågvar, 1982) and a group of predators or a disease may kill a large number of them, but the amount of fatty acids is obviously higher than would be expected to originate from collembolans in most natural environments. Therefore, linoleic acid was tested at two lower and more biologically realistic

doses. The threshold for the response was between the dose corresponding to 5 and 50 individuals (Figure 5). However, three crushed individuals of conspecifics were enough to evoke repellence (Nilsson and Bengtsson, 2004), in combination with avoidance of the aqueous phase of the two-phase extraction (Table 1) that points towards the use of a multitude of endogenous repellent substances.

Fatty acids initiate aggregation of other insects (McFarlane et al., 1983; Fuchs et al., 1985). If palmitic acid were responsible for the observed attraction of *P. armata* to live conspecifics (Nilsson and Bengtsson, 2004), one would expect an attraction also to *O. circulans* and *O. scotarius*, which contain higher concentrations of palmitic acid (Table 2). *P. armata* was attracted to traces of live *O. circulans* and *O. scotarius*, but the effect was not significant (Figure 1). The response to palmitic acid may also be interpreted as an attraction to a food source as discussed above.

2,3-Dimethoxy-pyrido[2,3-b]pyrazine is known to function as a predator defense in another Collembola, *Tetrodontophora bielanensis* (Waga) (Dettner et al., 1996), and it is possible that it makes *O. scotarius* and *O. circulans* repellent to their predators as well. The pyrido-pyrazine could also be an alarm substance. It is interesting to note that the same pyrido-pyrazine is found in a species of the Tetrodonthophorinae subfamily, *T. bielanensis*, in two species of the Onychiurinae subfamily, *O. scotarius* and *O. circulans*, but not in a third, *P. armata*. The compound may have developed separately in the two subfamilies or it is an ancient trait that has been lost in one species of the Onychiurinae subfamily. Alternatively, the morphology-based phylogenetic relationship between the species may not be correct.

P. armata is a pest to sugar beet seeds, which are treated with insecticides in commercial cultivation. A repellent substance, such as linoleic acid, may be a nontoxic and biodegradable alternative to traditional insecticides, especially if it is active against more than one species. Wild potato use aphid alarm pheromone to repel aphids (Gibson and Pickett, 1983), but examples of repellent semiochemicals used in pest management by humans are few (Jones, 1998).

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OVIPOSITION DETERRENTS IN LARVAL FRASS OF FOUR *Ostrinia* SPECIES FED ON AN ARTIFICIAL DIET

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Abstract—Behavioral bioassays have shown that volatile oviposition-deterring chemicals are present in the frass of *Ostrinia zealis*, *O. furnacalis*, *O. scapulalis*, and *O. latipennis* larvae fed on an artificial diet. These chemicals were extractable with acetone, and could be partitioned into a polar lipid fraction. This fraction mainly included palmitic, stearic, oleic, linoleic, and linolenic acids. No significant differences among the four *Ostrinia* species were found in the amount and composition of these free fatty acids. A mixture of the five authentic fatty acids of the composition found in the larval frass of *O. zealis* exhibited significant oviposition-deterring effects on all four species.

Key Words—*Ostrinia*, larval frass, oviposition deterrents, fatty acids.

INTRODUCTION

In many insect species, chemicals contained in larval frass deter oviposition of conspecific females. The ecological significance of these deterrents is to avoid competition among conspecific larvae for food by claiming pre-occupation of the host. Deterring effects of larval frass have been verified in both phytophagous and entomophagous insects. The former includes lepidopterans such as yellow cutworm *Agrotis segetum* (Anderson and Lofqvist, 1996), pineapple borer *Thecla basilides* (Rhainds et al., 1996), Egyptian cotton leaf worm *Spodoptera littoralis* (Hilker and Klein, 1989; Klein et al., 1990; Anderson et al., 1993), fall armyworm *S. frugiperda* (Williams et al., 1986), cabbage looper *Trichoplusia ni* (Renwick and Radke, 1980), European corn borer *Ostrinia nubilalis* (Dittrick et al., 1983),

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and coleopterans such as *Monochamus alternatus* (Anbutsu and Togashi, 2002). The oviposition deterrence of larval frass has been reported recently in two entomophagous ladybird beetles, *Harmonia axyridis* and *Propylea japonica* (Agarwala et al., 2003). In all species listed above, larval frass decreased oviposition of conspecific females significantly, with the highest reduction rate of about 90% in *A. segetum* (Anderson and Lofqvist, 1996) and *O. nubilalis* (Dittrick et al., 1983).

Chemical identification of oviposition deterrents is a primary step for further studies to explore the mode of communication, to infer the evolution of deterrents, and to develop applications in pest control. Reports on this aspect, however, are few. To the best of our knowledge, there has been only one paper on the chemical identification of deterrents in larval frass; a mixture of six compounds, benzaldehyde, carvacrol, eugenol, nerolidol, phytol, and thymol, was identified as an oviposition deterrent in frass of *S. littoralis* larvae fed on cotton leaves (Klein et al., 1990).

In the genus *Ostrinia* (Crambidae), eight species, *O. furnacalis*, *O. scapulalis*, *O. orientalis*, *O. zealis*, *O. zaguliaevi*, *O. palustralis*, *O. ovalipennis*, and *O. latipennis*, currently inhabit Japan (Ishikawa et al., 1999; Ohno, 2003), and the majority of them are cultured in our laboratory. Among these species, *O. latipennis* (knotweed borer) and *O. zealis* (burdock borer) are both oligophagous, but their host plant ranges differ largely; *O. latipennis* usually feeds on knotweeds *Reynoutria* spp. (Polygonaceae), whereas *O. zealis* feeds on Compositae plants such as burdock *Arctium lappa*, and thistles *Cirsium* spp. (Ishikawa et al., 1999). *O. furnacalis* (Asian corn borer) is known as an important pest of maize in Asia, but this species is actually polyphagous and feeds on various plants such as ginger *Zingiber officinalis* (Zingiberaceae), docks *Rumex* (Polygonaceae), and cocklebur *Xanthium* (Compositae) (Hattori and Mutuura, 1987). *O. scapulalis* (adzuki bean borer) is also polyphagous and feeds on many plants such as leguminous crops, hop *Humulus lupulus* (Moraceae), cockleburs *Xanthium*, and docks *Rumex*, but *O. scapulalis* is distinct from *O. furnacalis* in that it does not feed on maize (Hattori and Mutuura, 1987; Ishikawa et al., 1999). As exemplified by these four species, host plant preference in *Ostrinia* is diverse.

As mentioned above, European corn borer *O. nubilalis*, which is not found in the Far East, is known to produce oviposition deterrents in larval frass (Dittrick et al., 1983). Thus, *Ostrinia* species in Japan offer potential material for comparative studies on oviposition deterrents in larval frass. We addressed the following questions: (1) Do Japanese *Ostrinia* species produce oviposition deterrents in the frass? and (2) What are their interspecific effects? We report findings on oviposition deterrents from the larval frass of four *Ostrinia* species with different host plant preference, *O. latipennis, O. zealis, O. furnacalis*, and *O. scapulalis*.

METHODS AND MATERIALS

Insects and Frass Collection. Female adults of *O. scapulalis* and *O. furnacalis* were collected in the field at Matsudo (35.8°N, 139.9°E), Japan in June

and August 2002, respectively. *O. zealis* females were netted at Kawaji (36.9°N, 139.7°E) in July 2002. Egg masses of *O. latipennis* were collected from leaves of the giant knotweed *Reynoutria sachalinensis*, at Towa (39.9°N, C14:1.2°E) in July 2002. Larvae were reared as broods in rearing jars (8-cm diam) filled with 200 g of commercial diet for insects (Insecta LF, Nosan Corp., Yokohama, Japan). The environmental conditions were 24 ± 1°C, 15:9 hr L:D photocycle regime, and 60% RH. Females and males were separated during the pupal stage based on the morphology of the terminal abdominal segments. Mated females were obtained by introducing several pairs of 1- or 2-day-old males and females into a fabric screen cage (20 × 20 × 20 cm) and allowing them to mate for 2 nights. To minimize inbreeding, females from one family were put with males from another in each screen cage.

Fifth-instar larvae were confined in plastic dishes (10-cm diam) containing pieces of fresh artificial diet (Insecta LF) and maintained in a rearing chamber under the conditions described above. Larval frass was collected daily and stored in 50-ml glass bottles at −20°C until use. The common artificial diet was fed to the larvae of four species to facilitate comparison of deterrents among species; otherwise differences would be obscured by the natural variations in chemical compositions of the host plants. The precise composition of the artificial diet is not made public, but it is reported to contain mulberry leaf powder, soybean protein, starch, sugar, cereals powder, minerals, vitamins, citric acid, agar, and food antiseptics (water 72–76%; fiber <3.9%; protein >6.0%; ash <3.9%; fat <1.1%) (brochure from the supplier, Nosan Corp.).

Chemicals and Preparation of Test Materials. *n*-Heptadecane (>99%) was purchased from Tokyo Kasei Kogyo Co., Japan. Hexadecanoic acid (palmitic acid, $C_{16:0}$), octadecanoic acid (stearic acid, $C_{18:0}$), (*Z*)-9-octadecenoic acid (oleic acid, $C_{18:1}$), (*Z*,*Z*)-9,12-octadecadienoic acid (linoleic acid, $C_{18:2}$), and (*Z*,*Z*,*Z*)-9,12,15-octadecatrienoic acid (linolenic acid, $C_{18:3}$) were purchased from Sigma (St. Louis, MO).

Distilled water and analytical grade organic solvents were used to prepare the following test materials: (1) a water suspension of *O. zealis* larval frass, (2) a water suspension of fresh artificial diet, (3) an acetone extract of *O. zealis* larval frass, (4) a hexane-soluble fraction, which was obtained by drying the acetone extract under a stream of nitrogen, redissolving it in hexane, and filtration, and (5) a residual fraction, which was made by dissolving the residue on the filter in acetone. The hexane-soluble fraction was loaded on a Sep-Pak®Plus NH_2 cartridge (Waters, Milford, MA), and neutral (6) and polar (7) lipids were eluted with a 2:1 mixture of chloroform and 2-propanol (20 ml), and 2% acetic acid in diethyl ether (20 ml), respectively.

Bioassay. Bioassays were carried out in the environmental conditions outlined above. Before the test, about 20 pairs of newly emerged females and males were placed in a fabric screen cage (20 × 20 × 20 cm) for arbitrary mating for 2 nights to obtain mated females for the bioassay. After each experiment, the

bursa copulatrix was removed from each female and dissected to check for the presence of a spermatophore. Only those data obtained using mated females that had at least one spermatophore were used for statistical analyses.

All bioassays were carried out using a dual-choice test in the fabric screen cage. Test solution/suspension equivalent to 100 mg of fresh frass and the same amount of solvent were, respectively, applied to cut halves of a filter paper circle (90-mm diam). After evaporation of the solvent, the halves were taped together and stapled on the ceiling of the fabric screen cage 2 hr before the start of scotophase. Solid larval frass (100 mg) of the four *Ostrinia* species was bioassayed directly by sandwiching it between the layers of a piece of cotton ($4 \times 4 \times 0.1$ cm). A blank piece of cotton was used as the control.

Only one mated female was allowed to oviposit in each fabric screen cage to facilitate the checking of mating status, and also to avoid any possible influence on oviposition by other females. The following morning, the eggs (*O. zealis*) or egg masses (*O. furnacalis*, *O. scapulalis*, and *O. latipennis*) laid on each half of filter paper or piece of cotton were counted. *O. zealis* females usually laid 30–60 eggs, and females of the other species laid 3–6 egg masses on the following night after copulation.

Chromatography and Chemical Identification. Polar lipid fractions from larval frass of the four *Ostrinia* species and from fresh diet were dried under a stream of nitrogen and redissolved, respectively, in hexane containing 1 ng/μl of *n*-heptadecane as an internal standard. An aliquot (2 μl) of 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-35, 30 m $\times$ 0.25 mm i.d., film thickness 0.25 μm, J&W Scientific, Folsom, CA). 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-35: 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 carrier gas was nitrogen. The quantity of each compound was calculated on the basis of the peak area and calibrated by comparing it with that of heptadecane.

The polar lipid fractions were analyzed by GC–mass spectrometry (GC–MS) directly or after derivatization with *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) according to the method of Shepherd et al. (1995). A QP-5050A GC–MS (Shimadzu) system equipped with a DB-Wax or DB-35 column was run under the same temperature conditions as described for GC analysis. Helium was used as 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.

Fatty acids were tentatively identified by matching their mass spectra with those in the NIST Library using the software CLASS-5000 (Shimadzu), and further

verified by comparison of the diagnostic ions and the GC retention time with those of the respective authentic standard.

Statistical Analysis. In all tests, the number of eggs or egg masses for control (C) and treatment (T) were summed respectively for each replicate. The result is presented as an avoidance index (Ai): $\text{Ai} = (C - T)/(C + T)$ (Renwick and Radke, 1980). $\text{Ai} = 1$ indicates complete rejection of the test material. The null hypothesis that equal number of eggs or egg masses were laid for control and treatment ($\text{Ai} = 0$) was examined using the t test at $\alpha = 0.05$. Differences in the total amount of fatty acids in the frass of four *Ostrinia* species were examined with a one-way ANOVA.

RESULTS

Oviposition-Deterring Effect of Larval Frass. The water suspensions of *O. zealis* larval frass significantly deterred oviposition of conspecific females (FS in Figure 1). The water suspension of fresh artificial diet (DS) had a similar effect but the avoidance index was substantially lower. When exposed to the halves of filter paper treated with larval frass and fresh diet simultaneously, ovipositing females tended to select the latter significantly (Figure 1).

Larval frass from the four *Ostrinia* species sandwiched between the layers of a piece of cotton significantly deterred conspecific females from ovipositing

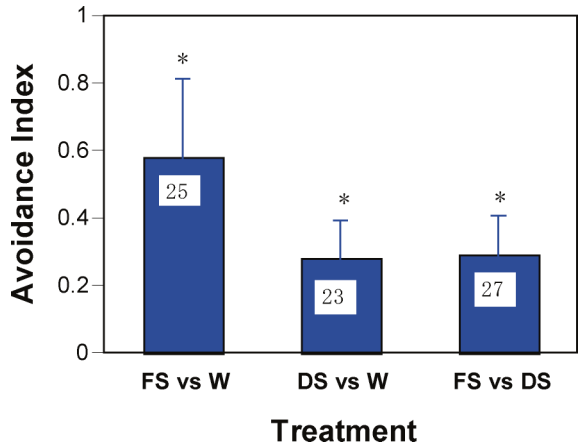

FIG. 1. Oviposition-deterring effect of water suspensions of *O. zealis* larval frass (FS) and artificial diet (DS) in dual-choice experiments. One milliliter of suspension (100 mg/ml) and water (W) was applied to a pair of halves of filter paper. See text for oviposition avoidance index. Values represent the mean ± SD. Numbers of replicates are shown inside the bars. *significantly different from 0 (t test, $P < 0.05$).

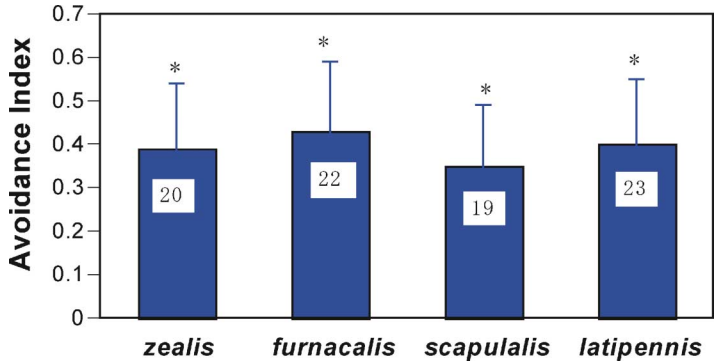

FIG. 2. Oviposition-deterring effect of larval frass from four *Ostrinia* species on respective females in dual-choice experiments using a pair of square cotton pieces ($4 \times 4 \times 0.1$ cm): a piece sandwiching 100 mg of larval frass was tested against a blank piece. See text and the legend to Figure 1 for further details.

(Figure 2). The avoidance index of the frass was similar among the four species (0.39–0.43). The avoidance indexes of the fresh and stored (at -20°C) frass were also not significantly different (data not shown).

Fractionation of Active Chemicals. The acetone extract of *O. zealis* larval frass deterred oviposition of conspecifics (AE in Figure 3). Both hexane-soluble and residual fractions had significant oviposition-deterring effects. However, when the two fractions were offered simultaneously, females preferred ovipositing on the residual fraction (HF and RF in Figure 3). When the hexane-soluble fraction was further fractionated into neutral and polar lipid fractions, the latter deterred oviposition more strongly (NL and PL in Figure 3).

GC-MS Analyses. The polar lipid fraction from *O. zealis* larval frass and that from the artificial diet were analyzed by GC and GC-MS (Table 1). The main components in larval frass were five free aliphatic fatty acids, palmitic, stearic, oleic, linoleic, and linolenic acids. Although the fresh artificial diet also contained these acids, their total amount was 3.3 times less than that in the frass (Table 1). Compositions of these fatty acids were similar among the four *Ostrinia* species (Figure 4). Linoleic acid was most abundant, followed by palmitic, linolenic, and oleic acid. Stearic acid was the least abundant. Total amounts of the fatty acids produced by the four *Ostrinia* species were 110–180 μg/100 mg fresh frass, and did not vary significantly among species (ANOVA, $P > 0.05$).

Effects of Authentic Free Fatty Acids. The oviposition-deterring effect of a synthetic mixture of the five fatty acids was tested at the ratio found in *O. zealis* larval frass. The mixture significantly deterred oviposition in the four *Ostrinia* species (Figure 5).

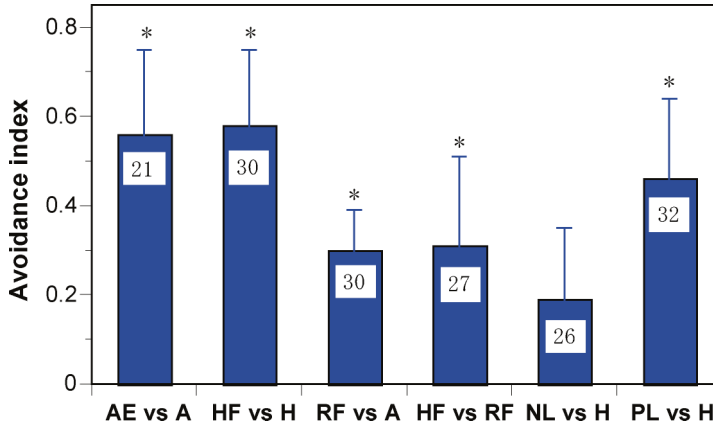

FIG. 3. Oviposition-deterring effect of fractions of *O. zealis* larval frass extract in dual-choice experiments. Extracts or fractions (equivalent to 100 mg of fresh frass) and corresponding solvents were, respectively, applied to a pair of halves of filter paper. AE, A, HF, H, RF, NL, and PL indicate acetone extract, acetone, hexane-soluble fraction, hexane, residual fraction, neutral lipids, and polar lipids, respectively. See text for hexane-soluble fraction, residual fraction, neutral lipids, and polar lipids. See the legend to Figure 1 for further explanations.

DISCUSSION

The present study has shown that larval frass from four *Ostrinia* species fed on an artificial diet exhibited significant oviposition-deterring effects. Compared with the avoidance index exhibited in *A. segetum* (Anderson and Lofqvist, 1996) and *O. nubilalis* (Dittrick et al., 1983), which was as high as 0.8, the values in our tests were low, 0.28–0.55. However, the lower avoidance index is partly attributable to the smaller amount of larval frass used for the bioassay in the present study (0.1 g

TABLE 1. AMOUNT OF CHEMICALS IN THE POLAR LIPID FRACTION FROM *O. zealis* FRASS AND IN FRESH DIET

Chemical	Diagnostic ions of trimethylsilyl derivative	μg/100 mg	
		Frass of *zealis*	Fresh diet
Palmitic acid	328(M), 313, 145,132, 129, 117, 75, 73	27.8	12.9
Stearic acid	356(M), 341, 145,132, 129, 117, 75, 73	13.9	1.3
Oleic acid	354(M), 339, 145,132, 129, 117, 75, 73	20.9	3.3
Linoleic acid	352(M), 337, 145,132, 129, 117, 75, 73	52.8	19.8
Linolenic acid	350(M), 335, 145,132, 129, 117, 75, 73	23.6	4.4
Total		139.0	41.7

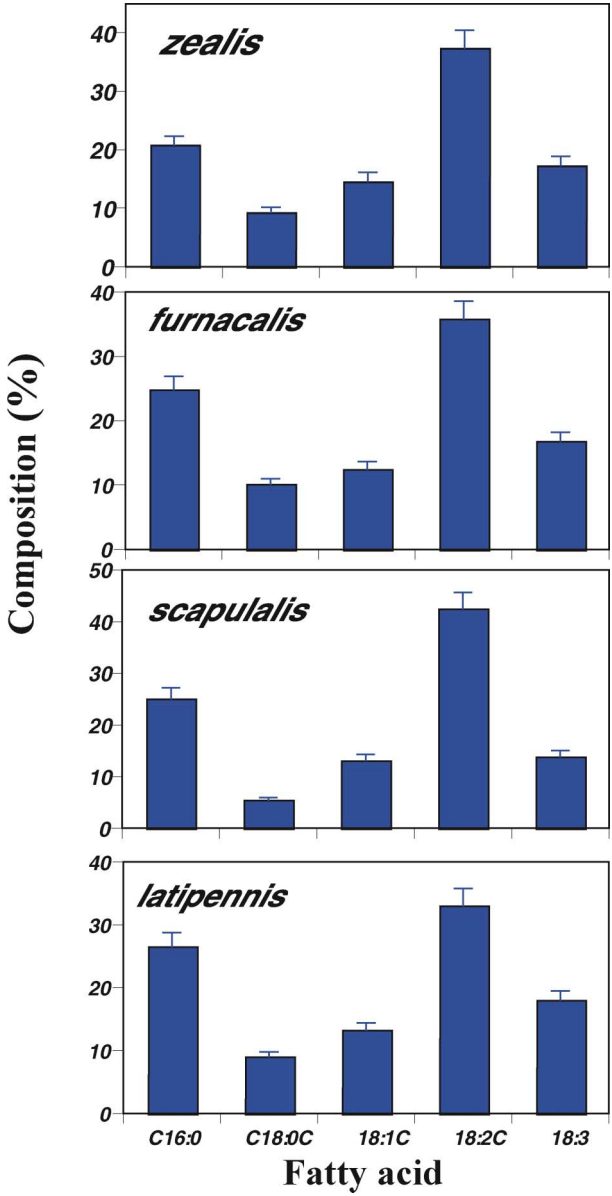

FIG. 4. The compositions of fatty acids in the polar lipid fraction from larval frass of four *Ostrinia* species analyzed by GC. $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$ represent palmitic, stearic, oleic, linoleic, and linolenic acids, respectively. Values represent the mean ± SD for four replicates (100% = total of the five fatty acids).

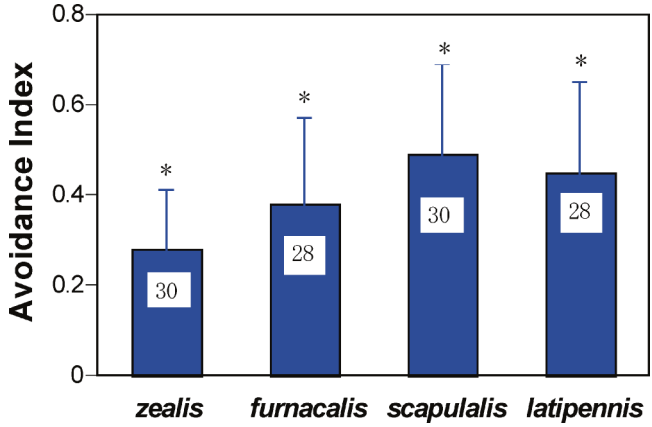

FIG. 5. Oviposition-deterring effect of a mixture of authentic fatty acids of the composition found in larval frass of *O. zealis* on four *Ostrinia* species. A hexane solution of fatty acids (150 μg) or hexane was applied to a pair of halves of filter paper. See text and legend to Figure 1 for further explanations.

fresh weight equivalent). In *O. nubilalis* and *A. segetum*, the highest reduction rate was obtained when 0.3 g and 0.6 g dry weight frass equivalent was used, respectively.

When an ovipositing female of *O. zealis* was exposed to the filter paper treated with the water suspension of larval frass, it may have perceived the frass powder visually, or perceived its texture and/or the chemicals in it after making contact. However, the obvious deterring effects of sandwiched frass demonstrated that the deterrence is mostly attributable to volatile chemicals.

Smaller amounts of oviposition-deterring fatty acids were found in fresh artificial diet. This accounts for the significant but lower oviposition deterrence of the fresh diet as compared with the larval frass. Larger amounts of the relevant fatty acids in frass as compared with fresh diet suggest that these acids are actively secreted by the larval gut. Analysis of the frass of larvae fed on an artificial diet free of these fatty acids should be useful in verifying this possibility.

The avoidance index of the mixture of the five fatty acids was lower than that of the acetone extract, hexane-soluble fraction, or polar lipid fraction, suggesting that some unidentified chemicals have oviposition-deterring or synergistic effects. Further effort is necessary to identify these deterrents and/or synergists. In parallel, the relative deterrence of the five fatty acids must be determined to elucidate structure–activity relationships.

Interestingly, similar aliphatic fatty acids have been identified as oviposition deterrents on the surface of egg masses of *O. scapulalis* and *O. furnacalis* (Li and

Ishikawa, unpublished). Moreover, oviposition-deterring effects of aliphatic fatty acids or their methyl esters have been reported in several species in Lepidoptera, Coleoptera, and Diptera. Gabel and Thiery (1996) demonstrated that $C_{14:0}$ (myristic acid), $C_{16:0}$, $C_{16:1}$ (palmitoleic acid), $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$ were the main deterrents in *Lobesia botrana* eggs. From *Cydia pomonella* eggs, $C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$ were identified as oviposition deterrents (Thiery et al., 1995). In *Helicoverpa armigera*, $C_{16:0}$ and $C_{18:1}$ were identified as oviposition deterrents (Li et al., 2001). Methyl esters of $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, and $C_{18:2}$ were reported to be oviposition deterrents from eggs of *O. nubilalis* (Thiery and Le Quere, 1991). In Coleoptera, the main oviposition deterrent from *Callosobruchus maculates* was $C_{18:1}$ (Sakai et al., 1986). Similarly, $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$ were identified in extract of the seventh urotergite gland, the secretory organ of oviposition deterrents, of *Ceutorhynchus assimilis* (Mudd et al., 1997). Moreover, $C_{18:1}$ was the most effective repellent to ovipositing *Culex quinquefasciatus* among *Z*-9-alkenoic acids (Hwang et al., 1984). Thus, straight chain fatty acids, especially $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$, seem to have broad repelling and deterring effects on oviposition. Why do so many insect species belonging to different orders use similar fatty acids as oviposition deterrents? There should be some causality behind this phenomenon. Further studies are necessary to shed light on this issue.

A question that must be addressed is the effectiveness of these fatty acids in the field. Although the larvae of *Ostrinia* usually feed inside the stem of host plants, most frass produced is conveyed outside through a hole made on the stem and often built up near it. Therefore, it is reasonable to assume that female adults perceive the oviposition deterrents emanating from the frass when they fly around the host plants for egg laying. However, this needs to be verified with field experiments.

Common usage of the same fatty acids as oviposition deterrents among the four *Ostrinia* species suggests that this trait was acquired in a common ancestor. It would be interesting to investigate whether all extant *Ostrinia* (21 species worldwide; Mutuura and Munroe, 1970; Ohno, 2003) have similar fatty acids in their larval frass. Ecologically, since pre-occupation of a host plant by larvae, including different species, suggests strong competition for food, avoiding oviposition in response to common chemical signals is probably adaptive for an *Ostrinia* female. In fact, several host plants are commonly utilized by multiple *Ostrinia* species (Ishikawa et al., 1999; S. Ohno and Y. Ishikawa, unpublished), and, thus, competition between heterospecific larvae can occur in nature.

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SEX PHEROMONES AND REPRODUCTIVE BEHAVIOR OF *Spodoptera litura* (FABRICIUS) MOTHS REARED FROM LARVAE TREATED WITH FOUR INSECTICIDES

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Abstract—When *Spodoptera litura* (Fabricius) larvae were treated with the insecticides deltamethrin, endosulfan, malathion, and carbaryl at dosages causing 30% mortality (LD_{30}), female moths reared from survivors showed similar patterns in the frequency of calling behavior. However, females reared from larvae dosed with deltamethrin had significantly higher titers of sex pheromone than those treated with endosulfan, malathion, or carbaryl, or control individuals. The ratio of the sex pheromone blend, *Z*9,*E*12-14:OAc to *Z*9,*E*11-14:OAc in females from the deltamethrin treatment group differed significantly from that of female moths in other treatments. In a wind-tunnel bioassay, males reared from larvae treated with deltamethrin, malathion, or carbaryl were less likely to display behaviors resulting in the location of a sex pheromone source than males reared from larvae treated with endosulfan or a solvent control (acetone). Mating success was significantly decreased in pairs containing either males or females reared from larvae that survived treatment with deltamethrin.

Key Words—*Spodoptera litura*, insecticide, sex pheromone, wind tunnel, reproductive behavior.

INTRODUCTION

Insecticides have been widely used for more than 50 years. Although the goal of insecticide application is to kill all target pests, survivors are common as target species develop resistance to particular compounds. Little is known of the

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physiology and behavior of these survivors, which may or may not differ from untreated individuals. Of particular importance is any alteration in physiology and/or behavior that might affect reproduction of survivors and their progeny.

Insecticide-treated males of the pink bollworm moth (*Pectinophora gossypiella* (Saunders)) and the oriental fruit moth (*Grapholita molesta* (Busck)) have reduced ability to locate sources of their sex pheromones (Floyd and Crowder, 1981; Linn and Roelofs, 1984; Haynes and Baker, 1985; Haynes et al., 1986). Sublethal permethrin treatment reduced the incidence of calling behavior of female pink bollworms (Haynes and Baker, 1985), as did cypermethrin treatment of cabbage looper moth females (*Trichoplusia ni* (Hübner)) (Clark and Haynes, 1992a). Chlordimeform at a dose of LD_1 decreased the mating success of males, but stimulated calling behavior and sex pheromone emission rates of female cabbage loopers (Clark and Haynes, 1992b). Recently, Yang and Du (2003) reported that topically applied sublethal doses of deltamethrin may interrupt chemical communication between male and female Asian corn borers (*Ostrinia furnacalis* (Güenee)) by affecting calling behavior. Deltamethrin treatment of the Asian corn borer females also decreased sex pheromone titers and PBAN (pheromone biosynthesis activating neuropeptide)-like activity. Previous studies have focused on sublethal effects of insecticides on the adult stage of lepidopteran pests. Here we focus on the effects of insecticide treatments applied to the larval stage of a major lepidopteran pest, the tobacco budworm, *Spodoptera litura* (Fabricius). Larvae of this insect feed on a wide spectrum of agricultural and horticultural crops, and as a result, many insecticide treatments target this pest. Here we examine the effects of application of pesticides to larvae on the sex pheromones and reproductive behaviour of survivors reaching adulthood.

METHODS AND MATERIALS

Insect Rearing and Pheromone Extraction. Larvae of *S. litura* were reared on artificial diet (Li et al., 1991) and pupae were separated by sex. Male and female moths were held in screened wooden cages 40 × 20 × 15 cm in separate environmental chambers (14L:10D, 25 ± 1°C) and fed 10% sucrose water solution. The ovipositors and sex pheromone glands were excised from 3-d-old virgin female moths during their peak calling period, and soaked in 10 μl of redistilled hexane for 30 min to extract pheromone components.

GC Analysis. Pheromone extracts were analyzed with a Hewlett-Packard 5890 gas chromatograph fitted with a HP-5 column (25 m × 0.2 mm ID) and a flame ionization detector. The oven temperature was maintained at 100°C for 2 min, and programmed at 10°/min to 220°C. Nitrogen was used as carrier gas. An Agilent 6890N-5973 GC-MS was used to confirm pheromone component identifications. The titer and the ratio of the pheromone components were calculated based on the

peak areas and calibrated versus an internal standard chemical, tridecyl acetate, 13:OAc.

Insecticide and Dosage. Organochlorine (endosulfan, 94.8% purity), organophosphate (malathion EC, 95% purity), carbamate (carbaryl, >99% purity), and pyrethroid insecticides (deltamethrin, 98%, Roussel Uclaf Corp., France) were chosen for study. Technical-grade insecticides were diluted in distilled acetone. Stock solutions of each insecticide were prepared at five concentrations (0.010, 0.025, 0.050, 0.250, and 0.500 ng/μl for deltamethrin; 0.500, 1.000, 2.500, 5.000, and 7.500 μg/μl for endosulfan, malathion, and carbaryl). Third instar larvae (16–17 mm in length) were dosed topically with 1 μl of insecticide solution on the dorsal side of the thoracic segment. Acetone was used as the control. After 24 hr, mortality for each group was recorded. Data were analyzed using probits (SPSS version 10.0, SPSS Inc., Illinois) and dosages resulting in 30% mortality (LD_{30}) were selected for further experimentation.

Calling Behavior. Calling behavior of virgin females was observed in a transparent glass cylindrical container (9 cm in length, 6 cm in diam) with a screen lid housed in an environmental chamber (25 $\pm$ 1°C, L14:D10 photoperiod) under dim red light. Observations were made on 30 females at 30 min intervals throughout the 10-hr scotophase. Calling behavior was defined as extrusion of the ovipositor and associated sex pheromone gland.

Copulation Success. Copulation success was measured using males and females that survived the deltamethrin treatment as larvae. Males and females were housed in different environmental chambers until they were paired. Four combinations of males and females were tested. One cylindrical carton (30 cm in length, 7 cm diam) was used to house a single pair. A rectangular board (5 $\times$ 6 cm) was inserted through the center of the carton. Two hours before initiation of scotophase, one male was introduced in one side of the carton and permitted contact with a virgin female in the other side of the carton by removing the divider. The cylinder was then put into a dark chamber for 10 hr. At the end of scotophase, the females were dissected and copulation was judged to have occurred if a spermatophore was present. Each combination was tested with 48 or more pairs.

Wind Tunnel Bioassay. The behavioral responses of adult males to sex pheromones were observed in a wind tunnel described by Tang et al. (1988). Males reared from the survivors of larval treatment with deltamethrin or acetone (control) were loaded into a cage (2.5 $\times$ 10 cm) on the second or the third scotophase after adult emergence. Thirty minutes before assays, caged males were transferred from the environmental chamber to the dark room that housed the wind tunnel. Assays were run between 7 and 9 hr after the initiation of scotophase under defined environmental conditions (0.3 lux of red lighting, temperature 25 $\pm$ 2°C, relative humidity 60–70%, and wind speed 0.3 m/sec). Males were released individually from a steel screen platform (28 cm above the floor of the wind tunnel) at the downwind end of the tunnel. Three hundred microgram of a blend of

(*Z*,*E*)-9,11-tetradecadienyl acetate (*Z*9,*E*11-14:OAc) and (*Z*,*E*)-9,12-tetradecadienyl acetate (*Z*9,*E*12-14:OAc) at the ratio of 9:1 was placed in a rubber septum as the pheromone source (Sun et al., 2002).

Male behaviors were recorded in the following categories: (1) taking flight, (2) stationary orientation (hovering near the release position), (3) upwind flight (oriented flight half way to the pheromone source), (4) approach to the pheromone source, and (5) contact with the pheromone source. At least 45 males were tested in each treatment (each male was tested once).

Statistical comparisons of the composition of pheromone components, male responses, and courtship interactions of males and females were performed using ANOVA (SPSS version 10.0, SPSS Inc., Illinois) followed by Duncan's new multiple range test. Percentage data were arc-sin square root transformed before analysis.

RESULTS

Sublethal Dosage. The dosages of endosulfan, malathion, carbaryl, and deltamethrin that yielded 30% mortality (LD_{30}) were 1.96, 2.43, 4.09 μg, and 0.03 ng, respectively. In subsequent experiments, third instars were topically treated with insecticides in 1 μl acetone solution (2.00 μg endosulfan, 2.50 μg malathion, 4.00 μg carbaryl, and 0.03 ng deltamethrin) at these concentrations; 1 μl of acetone was employed as a control.

Sublethal Effects of Insecticides on Calling Behavior. Calling behavior by females reared from survivors of insecticide treatments was similar to that of control females (Figure 1). At the peak period of calling, 8–9 hr after the initiation of scotophase, approximately 67% of control females, and 60, 63, 53, and 70% of females reared from larvae that survived deltamethrin, malathion, carbaryl, or endosulfan treatment showed calling behavior, respectively.

Sublethal Effects of Insecticides on the Ratio of Sex Pheromone Components. Four components, *Z*9,*E*11-14:OAc, *Z*9,*E*12-14:OAc, *E*11-14:OAc, and *Z*9-14:OAc were detected by GC analysis and confirmed by GC-MS in extracts from female pheromone glands. The percent composition of the four components in untreated females was 57.7, 14.6, 16.2, and 11.6%, respectively (Table 1). Adult females reared from larvae treated with endosulfan, malathion, or carbaryl had compositions of sex pheromones similar to those of control females ($P > 0.05$, Table 1). However, females reared from deltamethrin-treated larvae exhibited a different pattern. The relative percentage of *Z*9,*E*11-14:OAc was significantly lower than that in control females, whereas the relative percent compositions of the other three sex pheromone components were significantly higher than those of control females ($P < 0.05$, Table 1).

The ratio of *Z*9,*E*12-14:OAc to *Z*9,*E*11-14:OAc in deltamethrin-treated females was 0.83, whereas it was less than 0.34 in females treated as larvae with acetone or three other insecticides.

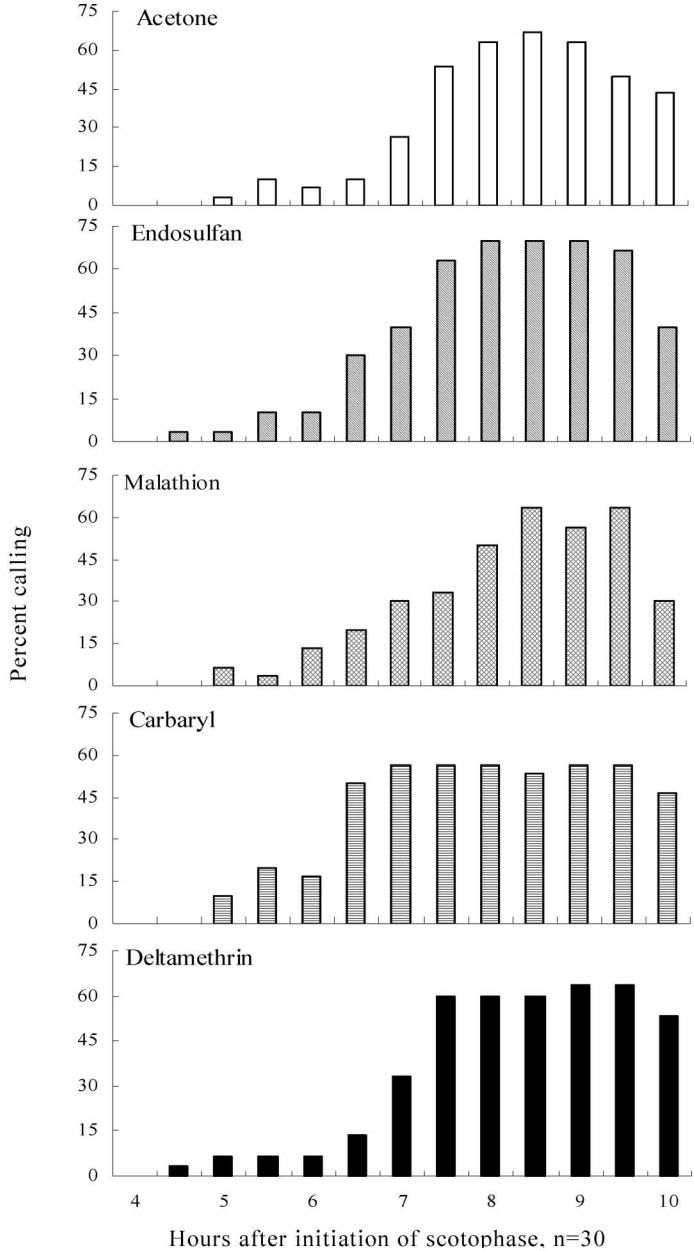

FIG. 1. Percent displaying calling behavior for female *Spodoptera litura* reared from survivors of larvae dosed with acetone or insecticides (data collected during third scotophase after adult emergence).

TABLE 1. MEAN PERCENT COMPOSITION (±SE) OF PHEROMONE COMPONENTS IN *Spodoptera litura* FEMALES REARED FROM UNTREATED INDIVIDUALS AND FROM SURVIVORS OF LARVAL TREATMENT WITH ACETONE OR INSECTICIDES

Treatment	Mean percent composition (±SE) of pheromone components				
	$A^{a,b}$	$B^{a,b}$	$C^{a,b}$	$D^{a,b}$	B/A^{a}
Untreated	57.7 ± 2.7 b	14.6 ± 1.6 a	16.2 ± 3.1 ab	11.6 ± 1.2 ab	0.25
Acetone	56.4 ± 1.4 b	18.8 ± 1.0 a	7.9 ± 1.4 a	16.8 ± 1.1 bc	0.33
Endosulfan	57.5 ± 2.2 b	14.4 ± 1.5 a	11.5 ± 2.8 a	16.6 ± 1.7 c	0.25
Malathion	56.3 ± 3.0 b	17.3 ± 1.2 a	16.7 ± 3.0 ab	9.7 ± 2.0 a	0.31
Carbaryl	53.7 ± 2.5 b	16.3 ± 2.3 a	10.9 ± 4.3 a	19.3 ± 2.0 cd	0.30
Deltamethrin	30.8 ± 2.6 a	25.4 ± 1.8 b	22.8 ± 2.2 b	21.0 ± 1.7 d	0.83

[a] *A*: *Z*9,*E*11-14:OAc; *B*: *Z*9,*E*12-14:OAc; *C*: *E*11-14:OAc; *D*: *Z*9-14:OAc; and *B*/*A*: ratio of *Z*9,*E*12-14:OAc/*Z*9,*E*11-14:OAc.
[b] Values in the same column followed by the same letter did not differ significantly at $P < 0.05$ (ANOVA followed by Duncan's new multiple range test).

Sublethal Effects of Insecticides on Sex Pheromone Titer. The titer of the four components of sex pheromones, *Z*9,*E*11-14:OAc, *Z*9,*E*12-14:OAc, *E*11-14:OAc, and *Z*9-14:OAc, in females reared from untreated, normal larvae averaged 6.8, 1.7, 1.9, and 1.4 ng, respectively (Table 2). There was no significant difference in the titers of the four components of sex pheromone among female moths reared from survivors of endosulfan, malathion or carbaryl treatment, or control individuals (Table 2). However, after treatment with deltamethrin in the larval stage, the mean amount of *Z*9,*E*12-14:OAc, *Z*9,*E*11-14:OAc, and *Z*9,*E*11-14:OAc in females was 20.6, 17.0, 15.3, and 14.1 ng/female, respectively, a significant increase compared with all other treatments ($P < 0.05$, Table 2). The increase in the titers of

TABLE 2. MEAN TITERS (ng ± SE) OF SEX PHEROMONE IN *Spodoptera litura* FEMALES REARED FROM UNTREATED INDIVIDUALS AND FROM SURVIVORS OF TREATMENT OF LARVAE WITH ACETONE OR INSECTICIDES

Treatment	No. of moths	Mean titers (ng ± SE)				
		$A^{a,b}$	$B^{a,b}$	$C^{a,b}$	$D^{a,b}$	Total titer[b]
Untreated	20	6.8 ± 0.7 a	1.7 ± 0.3 a	1.9 ± 0.3 a	1.4 ± 0.2 a	11.8 ± 1.1 a
Acetone	60	6.6 ± 0.6 a	2.2 ± 0.2 a	0.9 ± 0.2 a	2.0 ± 0.2 a	11.7 ± 0.9 a
Endosulfan	35	5.7 ± 0.7 a	1.4 ± 0.2 a	1.2 ± 1.9 a	1.7 ± 0.2 a	10.0 ± 1.2 a
Malathion	30	8.2 ± 1.8 a	2.6 ± 0.4 a	2.6 ± 0.3 a	1.5 ± 0.6 ab	15.3 ± 2.4 a
Carbaryl	37	5.8 ± 1.1 a	1.8 ± 0.3 a	1.2 ± 0.7 a	2.1 ± 0.4 a	10.9 ± 1.8 a
Deltamethrin	53	20.6 ± 6.6 b	17.0 ± 4.7 b	15.3 ± 5.8 b	14.1 ± 3.9 b	67.0 ± 13.1 b

[a] *A*: *Z*9,*E*11-14:OAc; *B*: *Z*9,*E*12-14:OAc; *C*: *E*11-14:OAc; and *D*: *Z*9-14:OAc.
[b] Values in the same column followed with the same letter were not significantly different at $P < 0.05$ (ANOVA followed by Duncan's new multiple range test).

TABLE 3. BEHAVIORAL RESPONSES (MEAN PERCENT ± SE) OF MALE *Spodoptera litura* TO SEX PHEROMONES IN WIND TUNNEL ASSAYS[a]

Treatment	No. of moths	Mean percent ± SE				
		Taking flight	Orientation[b]	Upwind flight[b]	Source approaching[b]	Source contact[b]
Acetone	60	100 a	85.4 ± 2.8 ab	65.5 ± 1.2 ab	57.1 ± 2.0 b	32.7 ± 1.0 c
Endosulfan	45	100 a	93.3 ± 6.7 b	86.7 ± 6.7 b	68.9 ± 9.7 b	33.3 ± 2.4 c
Malathion	45	95.6 ± 2.2 a	77.8 ± 2.2 a	53.3 ± 6.7 a	35.6 ± 4.4 a	24.4 ± 2.2 b
Carbaryl	45	95.6 ± 4.4 a	86.7 ± 3.8 ab	68.9 ± 5.9 ab	33.3 ± 1.4 a	13.3 ± 3.8 a
Deltamethrin	65	97.8 ± 2.2 a	79.4 ± 3.4 a	55.0 ± 10.8 a	34.4 ± 6.2 a	20.6 ± 3.4 ab

[a] Percentages in the same column followed with the same letter were not significantly different (ANOVA followed by Duncan's new multiple range test, $P < 0.05$).

[b] Orientation: hovering near the release position upwind flight: oriented flight half way to the pheromone source; source approaching: approaching to within 10 cm of the pheromone source; source contact: contact with the pheromone source.

those two components was responsible for the significant increase in total titer of sex pheromones documented (Table 2).

Sublethal Effects on Male Responses to Sex Pheromone. Treatment of larvae with four insecticides resulted in different effects on *S. litura* male responses to sex pheromones in the wind tunnel assays (Table 3). None of the insecticides affected the percent of males which displayed "taking flight" behavior. However, from "orientation" to "source contact" behavior, larval exposure to malathion, carbaryl, or deltamethrin decreased the capacity of *S. litura* males to locate the pheromone source as measured by decreases in percent response in one of more of the categories of upwind flight, source approach, or source cantact (Table 3).

Sublethal Effects of Deltamethrin on Mating Behavior. Treatment of larvae with deltamethrin reduced the mating success of adult survivors. In four treatment groups, control pairs (both sexes treated with acetone in the larval stage) were significantly more likely to mate successfully (74%) than pairs where either or both members had been treated with deltamethrin as larvae (43–52%, Table 4). There was no difference in the percentage of successful mating between pairs where only one member had been treated with insecticide as larvae as compared with pairs in which both members were treated.

DISCUSSION

Among the four insecticides tested, deltamethrin treatment (LD_{30} dose) at the larval stage reduced the ability of males to locate sex pheromone sources in the wind-tunnel bioassay, altered the percentage composition and titer of sex

TABLE 4. COPULATION SUCCESS OF MALE AND FEMALE *Spodoptera litura* REARED FROM INDIVIDUALS THAT SURVIVED LARVAL TREATMENT WITH ACETONE OR INSECTICIDE[a]

Treatment combination (Female × Male)	No. of pairs	Percent copulating ± SE[b]
Acetone × Acetone	54	74.2 ± 4.4 b
Acetone × Deltamethrin	50	43.3 ± 5.1 a
Deltamethrin × Acetone	48	45.8 ± 7.5 a
Deltamethrin × Deltamethrin	52	51.7 ± 8.3 a

[a] Copulation success was judged by the presence of a spermatophore in the female reproductive tract.
[b] Percentages followed with the same letter were not significantly different (ANOVA followed by Duncan's new multiple range test, $P < 0.05$).

pheromones in extracts of female sex pheromone glands, and decreased the mating success of both female and male *S. litura*. We found no evidence that malathion, carbaryl, or endosulfan treatment resulted in significant reduction in the titer or composition of *S. litura* sex pheromone.

In the current study, the amount and percentage composition of the four components of the sex pheromones of *S. litura*, the tobacco budworm, changed after larval treatment with deltamethrin. Tamaki et al. (1973, 1976) reported that the sex pheromone of tobacco budworms was composed of four compounds, *Z*9,*E*11-14:OAc, *Z*9,*E*12-14:OAc, *E*11-14:OAc, and *Z*9-14:OAc. *Z*9,*E*11-14:OAc and *Z*9,*E*12-14:OAc were proved to be the active components in the sex pheromone (Tamaki and Yushima, 1974; Yushima et al., 1974; Sun et al., 2002). The ratio of *Z*9,*E*11-14:OAc to *Z*9,*E*12-14:OAc in sex pheromone gland extracts was ~3:1 (Sun et al., 2002). Our results replicated this pheromone identification for control individuals and for adults reared from larvae treated with endosulfan, malathion, and carbaryl. In contrast, females reared from larvae treated with deltamethrin showed a different pattern, in which the ratio of the two major components was ~1:1. In addition, the titers of the minor components, *E*11-14:OAc and *Z*11-14:OAc, also increased significantly, to ~9 times the level of the controls. The mechanism underlying this dramatic change in pheromone titers and compositions is not known.

Compared to deltamethrin treatment of Asian corn borer adults, *O. furnacalis* (Yang and Du, 2003), treatment of *S. litura* larvae with the same insecticide resulted in a different pattern of variation in both sex pheromone ratio and titer. The ratio of *E*- and *Z*-isomers of 12-tetradecenyl acetate in Asian corn borer pheromone glands did not change, but the titer significantly decreased. At the 1st, 2nd, and 4th day posttreatment with deltamethrin at the LD_{30} dose, the sex pheromone titer of Asian corn borer females was 15, 20, and 25% the titer of control females. As a result, chemical communication between the sexes was significantly affected for at least 4 d after treatment. Both Asian corn borers and tobacco budworms are Lepidoptera,

it is not known why deltamethrin treatment resulted in different responses in the two species. One possible explanation is the period of development when treatments were applied (adult vs. larvae). Previous reports have suggested that sublethal treatments may affect insect behavior. Clark and Haynes (1992a,b) reported that cypermethrin or chlordimeform (a formamidine insecticide) treatment of adults decreased the mating success of male *Trichoplusia ni*. Here, we show that mating success was reduced in both male and female moths reared from survivors of treatment of larvae with deltamethrin.

In the wind tunnel, the proportion of males reaching the source of sex pheromone was reduced after treatment of larvae with deltamethrin. This result is similar to studies of the effects of cypermethrin and permethrin on males of *Pectinophora gossypiella* and *T. ni* (Linn and Roelofs, 1984; Haynes and Baker, 1985; Haynes et al., 1986; Clark and Haynes, 1992a). In the current study, carbaryl also decreased the percentage of male survivors that contacted the source of the sex pheromone, but endosulfan had no effects on male survivors when compared to controls. In contrast, hypersensitivity to sex pheromone was observed in male *Grapholita molesta* dosed with octopamine (Linn and Roelofs, 1986), and male *T. ni* treated with chlordimeform (Linn and Roelofs, 1985). Female *T. ni* treated with chlordimeform released more of the major pheromone component, *Z*7-12:OAc, than control females during the first 4 hr of scotophase, but emitted less pheromone later in the scotophase (4–8 hr) (Clark and Haynes, 1992b).

Sex pheromones of female Lepidoptera exhibit a high degree of specificity, and male responses are affected by changes in the quality and quantity of compounds in the odor plume (Roelofs and Jurenka, 1996). The current study showed that female moths reared from adult survivors of deltamethrin treatment of larvae had a different sex pheromone blend than control females and from females treated with three other insecticides. In addition, males reared from larvae that survived deltametrin treatment were less likely to contact a pheromone source than control males. Our results further confirmed the effects of sublethal treatment with insecticides on insect reproductive behavior. One implication is that reduced insecticide concentrations could result in decreases in pest populations because sublethal exposure may interfere with communication between male and female *S. litura* individuals. It is possible that other pest species may be similarly affected by sublethal exposure to pesticides. However, for practical purposes, use of sublethal doses of pesticides for disrupting reproductive behaviors of pests must be tested thoroughly before implemantation because of the risk of accelerating development of resistance.

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CHEMICAL INTERACTIONS OF *Brachiaria plantaginea* WITH *Commelina bengalensis* AND *Acanthospermum hispidum* IN SOYBEAN CROPPING SYSTEMS

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Abstract—Previous results obtained in soybean–wheat rotations under no-tillage conditions showed reductions in the seedbank of the weed species *Commelina benghalensis*, but no alteration in the seedbank of *Acanthospermum hispidum* in areas infested with *Brachiaria plantaginea*. Analyses of the soluble fraction of *B. plantaginea* indicated the predominance of aconitic acid (AA) among the aliphatic acids and ferulic acid (FA) among the phenolic acids. Laboratory bioassays using *C. benghalensis* and *A. hispidum* were carried out to evaluate phytotoxic effects of pure organic acid solutions and dilute extracts of *B. plantaginea* on seed germination, root development, and fungal germination. Solutions of AA and FA were prepared at 0.25, 0.50, and 1.0 mM. Extracts of *B. plantaginea* were diluted to obtain concentrations of AA similar to those in the prepared solutions. Seeds were sown on 0.5% agar (containing AA, FA, or diluted extract) in plastic-covered receptacles and maintained in a germination chamber for 10 days. AA and FA solutions and the *B. plantaginea* extract reduced germination and root length, mainly of *C. benghalensis*. AA also stimulated the development of endophytic fungi (*Fusarium solani*), which had complementary adverse effects on *C. benghalensis* germination. FA and AA may play important roles in reducing the seedbank of some weed species, acting directly on germination and development and, indirectly, by stimulating endophytic fungi that alter germination.

Key Words—Aconitic acid, ferulic acid, organic acids, allelopathy, *Commelina benghalensis*, *Acanthospermum hispidum*, *Brachiaria plantaginea*, *Fusarium solani*, weed seedbank, seed germination.

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INTRODUCTION

No-tillage agriculture improves not only soil and water conservation, but also benefits other variables that affect crop productivity. For instance, rotations in no-tillage systems can be an important tool for weed control, through competition or allelopathy, and release of organic compounds that influence weed establishment and development (Souza-Filho et al., 1997; Favero et al., 2001; Severino and Christoffoleti, 2001).

Competitiveness of weed species varies with their developmental characteristics, especially establishment rapidity, and the quantity and length of time residues are present on the soil surface. On the other hand, decomposing plant residues release low molecular weight compounds into the soil that vary in concentration and function with plant species, variety, nutritional status, and age (Jones, 1998; Franchini et al., 2003). Those organic compounds have functional groups, mainly carboxyls and phenols, that participate in many chemical reactions and may influence germination and development of competing plant species.

Several studies have shown that during plant-residue decomposition, compounds released include phenolic acids (ferulic, coumaric, and caffeic) that affect weed germination and seedling development, consequently influencing weed seedbanks (Guenzi and McCalla, 1966; Cochran et al., 1977; Liebl and Worsham, 1983).

Field experiments with various soybean-management practices, seeking to evaluate control of the weed species *Commelina benghalensis* L. (wandering Jew) and *Acanthospermum hispidum* DC. (hispid starburr), showed greater reductions in seedbanks of the former in the presence of high infestations of *Brachiaria plantaginea* (Link) A.S. Hitchc. (Alexander grass) than with chemical control measures (Voll et al., 1997a). Survival estimates for *A. hispidum* were independent of tillage method or chemical control (Voll et al., 1997b).

On the basis of these results, extracts of *B. plantaginea* were tested under laboratory conditions. The main organic compounds were identified, and the effects of solutions of organic compounds and diluted weed extracts on the germination and development of *C. benghalensis* and *A. hispidum* were evaluated.

METHODS AND MATERIALS

Extraction and Characterization of Brachiaria plantaginea. *B. plantaginea* shoots were collected from natural stands at the Embrapa Soybean Experiment Station in Londrina, Parana State, Brazil, in the summer of 2001. Plants were collected at physiological maturity and dried at 45°C in a forced-air oven. The material was homogenized by grinding, passed through a 2-mm sieve, and stored in dark-glass flasks at 4°C. For extraction of the soluble organic fraction, 60-g portions of material were dispersed in to 1-l aliquots of deionized water and mixed

at 200 rpm for 1 hr. The solution was passed trough a 0.5-mm sieve to remove most of the plant material. The pH was reduced to 2.5 with 0.1 N HCl, and the solution was centrifuged at 2500*g* for 15 min. The supernatant was extracted $\times 3$ with ethyl acetate. After volume reduction under reduced pressure, the residue was dissolved in 20 ml of 80% methanol and diluted 100-fold in the same solvent for analysis of phenolic and aliphatic organic acids (POA and AOA) by high-performance liquid chromatography, as described by Franchini et al. (2003).

For the analysis of phenolic acids, a reverse-phase column was used (ODS-C18, Shim-pack) with a mobile phase of 2% acetic acid (A) and methanol:acetic acid:water (18:1:1) (B). A linear gradient from 25 to 75% of B over 40 min was used (flow rate 1 ml min^{-1} and detection wavelength 260 nm). For the analysis of aliphatic acids, an ion-exchange column was used (HPX-87H, Bio-Rad) with a mobile phase of 0.005 M H_2SO_4 (flow rate 0.6 ml min^{-1} and detection wavelength 210 nm). Identification of the organic compounds was accomplished by comparison of retention times and the ultraviolet spectra of pure samples.

Bioassays with Prepared Solutions and Diluted Brachiaria plantaginea *Extracts*. The bioassays were developed in covered 500-ml plastic containers (polyethylene, cubic form, $10 \times 10 \times 5$ cm, $l \times w \times h$) containing 0.5% agar as the medium for dilution of pure compounds, *B. plantaginea* extracts, and for *C. benghalensis* and *A. hispidum* seed growth and germination. Into 1-l flasks, 4-g portions of agar were added to 800-ml aliquots of deionized water. The solutions were autoclaved for 1 hr and placed into a 40°C water bath. Aliquots of concentrated solutions of aconitic or ferulic acids were added to obtain concentrations of 0.25, 0.50, and 1.00 mM. As the concentrated *B. plantaginea* extract contained 20.25 mg ml^{-1} of aconitic acid, to obtain the same concentrations of pure solution, 1.75, 3.50, and 7.00 ml of the concentrated extract were added to diluted extracts. Agar preparations containing the pure solutions and diluted *B. plantaginea* extract were transferred into eight plastic receptacles (100 ml/receptacle) that constituted treatment replicates. Each agar layer was approximately 1-cm thick. The control treatment consisted of 0.5% agar only.

Commelina benghalensis and *A. hispidum* seeds collected in the summer of 2001 were surface-disinfected for 2 min with 2% sodium hypochlorite. Fifty seeds were sown per receptacle and transferred into a germination chamber with a 14/10 hr L/D photoperiod and temperatures of 30°C/20°C, respectively. After 10 d, germination rate (%), root length (mm), and the number of seeds infected by endophytic fungi (%) were determined. Colonies of fungi were collected and identified as *Fusarium solani*. Germination rates and root length were transformed into relative values according to the equation:

$$\text{Relative value} = \text{control value/treatment value}$$

Statistical Analyses. All data were analyzed using SAS (SAS Institute, 1996). The effects of treatments on each parameter were analyzed by analyses of variance

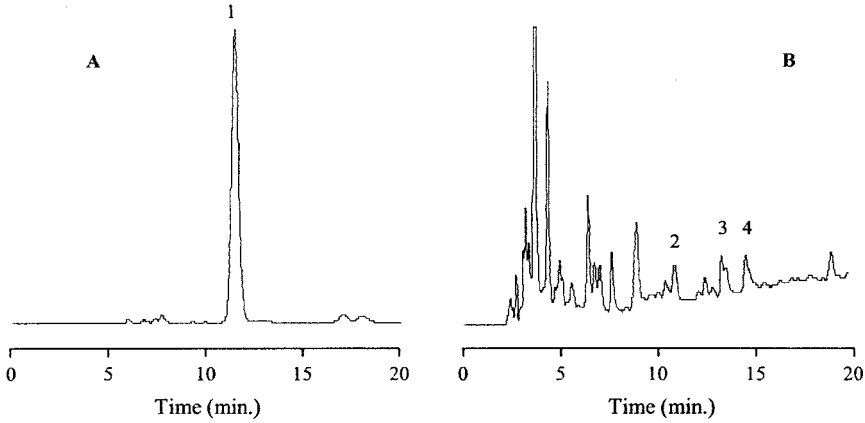

FIG. 1. HPLC chromatograms of organic acids in *B. plantaginea* extract. (A) Aliphatic acids and (B) phenolic acids. Numbers refer to the compounds shown in Table 1.

(ANOVA). If the ANOVA indicated a significant treatment effect at the 5% probability level ($P < 0.05$), the means were compared by Tukey's test.

RESULTS AND DISCUSSION

Figure 1 and Table 1 show chromatograms and parameters used for identification and quantification of the organic acids. Aconitic acid was predominant in *B. plantaginea* extracts; small amounts of phenolic acids were also present.

Seed germination and root length of the weed species were affected in different ways by the pure solutions and *B. plantaginea* extracts (Figure 2A and B). Aconitic acid stimulated *A. hispidum* and inhibited *C. benghalensis* seed germination. *B. plantaginea* extract inhibited seed germination of both species, especially

TABLE 1. PARAMETERS FOR IDENTIFICATION AND QUANTIFICATION OF ORGANIC ACIDS IN *B. plantaginea* EXTRACTS

Organic compound	Retention time (min)	Rate of extraction (%)	Concentration (g kg^{-1})	Equation	r^2
t-Aconitic acid	11.89	83 ± 4.4	6.752 ± 0.220	$y^a = 4.43$ E-08$x^b + 5.62$ E-03	1.000
Caffeic acid	10.98	84 ± 3.8	0.096 ± 0.008	$y = 1.58$ E-07$x + 7.40$ E-03	1.000
p-Coumaric acid	13.42	101 ± 4.8	0.123 ± 0.011	$y = 1.81$ E-07$x + 1.02$ E-02	0.998
Ferulic acid	14.67	101 ± 5.4	0.124 ± 0.010	$y = 1.64$ E-07$x + 9.42$ E-03	0.999

[a] Acid concentration (mM).
[b] Peak area.

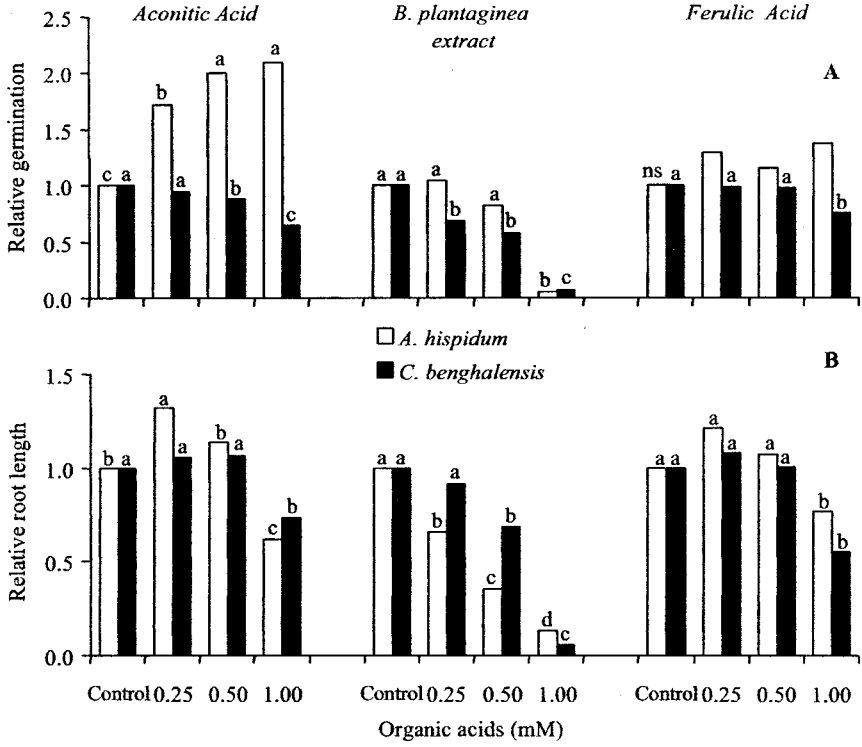

FIG. 2. Relative germination (A) and root length (B) of *C. benghalensis* and *A. hispidum* in solutions of organic acids and *B. plantaginea* extracts providing the indicated concentrations of aconitic acid. Different letters above columns mean significant differences (Tukey $P < 0.05$) within each plant species and extract/acids.

C. benghalensis. Ferulic acid had a smaller effect on seed germination, inhibiting the germination of *C. benghalensis* only with the highest dose (Figure 2A).

In general, root development was inhibited in both species, except for a stimulatory effect from an intermediate dose of aconitic acid on *A. hispidum* (Figure 2B). Root lengths of both species were more affected by *B. plantaginea* extracts and the effects were proportional to concentration. The pure solutions were inhibitory only at the highest concentration.

Fungal development on seeds was influenced by the presence of aconitic acid, whereas no significant effects were observed with ferulic acid (Figure 3). *C. benghalensis* had 7.3 times more seeds infested by fungi than did *A. hispidum*. The higher fungal development in *C. benghalensis* shows that its seeds had more fungal inoculum in relation to *A. hispidum*. The low control values with external seed disinfection suggests that the fungi are endophytic.

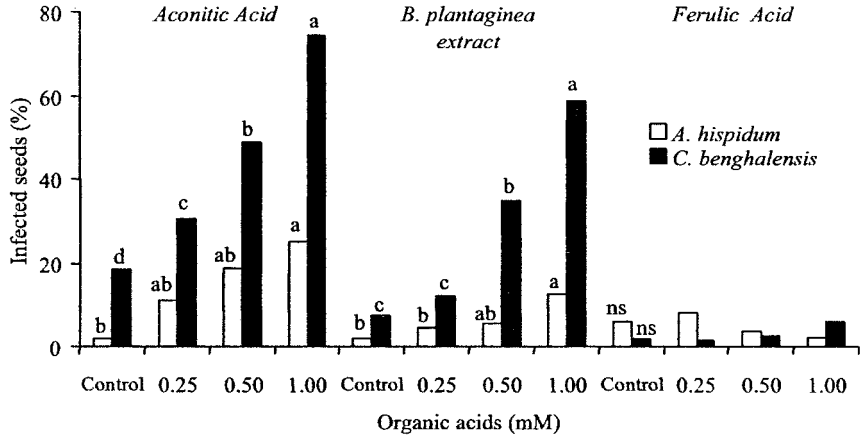

FIG. 3. Fungal infestation on *C. benghalensis* and *A. hispidum* seeds in solutions of organic acids and *B. plantaginea* extracts providing the indicated concentration of aconitic acid. Different letters above columns mean significant differences (Tukey $P < 0.05$) within each plant species and extract/acids.

In the presence of aconitic acid and *B. plantaginea* extract, fungal development in *C. benghalensis* seeds was inversely related to seed germination, suggesting that fungal growth adversely affects germination (Figure 4).

B. plantaginea extract was less effective than pure aconitic acid in terms of enhanced fungal infection, but more effective in terms of inhibiting germination

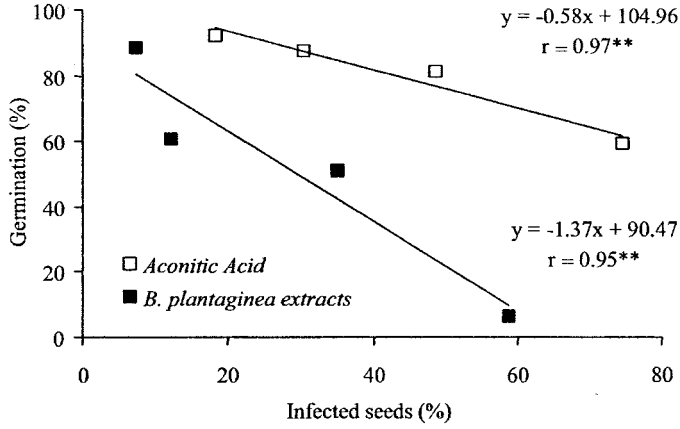

FIG. 4. Relationship between fungal infestation and *C. benghalensis* seed germination in pure solutions of organic acids and *B. plantaginea* extracts.

and plant growth, suggesting that different mechanisms are involved. The data in Figure 2 suggests that at least one other unidentified phytotoxic compound in *B. plantaginea* extract contributes to the germination and growth effects.

These results support those observed in the field, where *B. plantaginea* infestation had a greater effect on survival of *C. benghalensis* seedbanks in relation to those of *A. hispidum* (Voll et al., 1997a,b). This appears to be related to occurrence of endophytic fungi and responses to organic compounds released by *B. plantaginea*.

The presence of fungi (including *F. solani*) in the seeds of weedy plants, possibly acting as a dissemination medium and source of disease, is common (Redlin and Carris, 1996). Endophytic microorganisms penetrate plants via natural openings and wounds (e.g., in roots and through stomates) or by enzyme production or structures that facilitate penetration. Some endophytic microorganisms may also be transmitted in seeds (Azevedo, 1998).

The higher relative occurrence of endophytic fungi in *C. benghalensis* compared to *A. hispidum* implies greater susceptibility to factors that stimulate spore germination in seeds, as was the case for aconitic acid. The specific effect of aconitic acid on fungal biology needs to be evaluated. *Acanthospermum hispidum* may contain higher levels of endogenous fungicidal or fungistatic compounds that reduce fungal germination. Other important interactions associated with the development of fungi in seeds may also be involved. Hatzios (1987) observed that several species of *Fusarium* were able to produce fusaric acid in infected tomato and cotton plants. This product is chemically related to picloram, a commercial herbicide.

Aconitic acid is a component of the glyoxylate cycle and the tricarboxylic acid or Krebs's cycle (Goodwin and Mercer, 1983). Its presence has been reported in various plant species, including wheat (*Triticum aestivum*) (Burke et al., 1990; Thompson et al., 1997), sugar cane (*Saccharum officinalis*) (Hanine et al., 1990), quackgrass (*Agropyron repens*) (Friebe et al., 1995), and black oat (*Avena strigosa*) (Franchini et al., 2003). In terms of allelochemical effects, high concentrations of aconitic acid in barnyard grass have been associated with antinutritional effects on grasshoppers (Katsuhara et al., 1993; Watanabe et al., 1997).

Although aconitic acid is known to be involved in several biochemical pathways in plants, we believe that this is the first reported study on its effects on weed germination and development of endophytic fungi. In contrast, allelopathic effects of phenolic acids have been reported in the literature (Guenzi and McCalla, 1966; Cochran et al., 1977; Liebl and Worsham, 1983; Rice, 1984). The allelochemical effects of aconitic acid in the present study were equivalent or superior to those observed for ferulic acid on *C. benghalensis* seed germination and root development. Considering the high concentrations of aconitic acid in *B. plantaginea* (up to 6.75 g kg^{-1} of dry material) and its effects on germination, root growth, and development of endophytic fungi in *C. benghalensis* seeds, it was probably

the main agent responsible for reduced survival of weeds in the presence of high *B. plantaginea* infestation that was observed by Voll et al. (1997a).

A noteworthy aspect of this study is that *B. plantaginea* had no detrimental effects on germination and initial development of soybean, suggesting possible benefits when the legume is preceded by a grass pasture that includes *B. plantaginea*. Thus, maintaining *B. plantaginea* as a management strategy is particularly important for soybean when grown under no-tillage due to difficulties controlling *C. benghalensis* with herbicides.

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ERRATUM

FLORAL CO_2 REVEALS FLOWER PROFITABILITY TO MOTHS

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Due to errors by the Publisher, Fig. 1 and the reference IPCC 2001 in the above paper (which was originally published in Vol. 30, No. 6, June 2004) were incorrectly represented. The correct representations appear below.

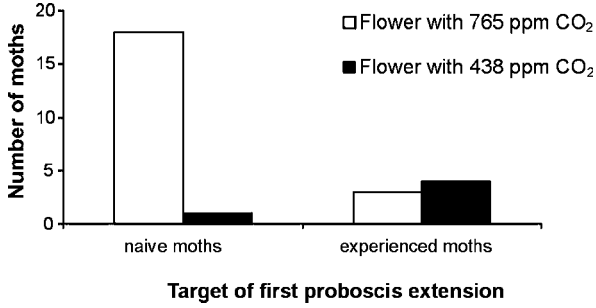

FIG. 1. Targets of the first proboscis extensions (feeding attempts) of naïve and experienced *Manduca* moths: number of moths targeting the surrogate flower with a high level of CO_2 as typical for newly opened *Datura* flowers (765 ppm, white bars), and an approximately ambient level of CO_2 (438 ppm, black bars). The position of flowers in the room did not affect the outcome of experiments. Only the first proboscis extension of a moth during each experiment was considered for analysis of foraging preference, as the first experience might affect successive foraging decisions.

Reference change:

IPCC. 2001. Climate Change 2001: The Scientific Basis (Report of Working Group 1 of the Intergovernmental Panel on Climate Change, IPCC Secretariat, Geneva, 2001).

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THE CHEMISTRY OF EXPLODING ANTS, *Camponotus* SPP. (*cylindricus* COMPLEX)

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Abstract—A detailed comparative analysis of the exocrine chemistry of nine Bruneian *Camponotus* species in the *cylindricus* complex is reported. Workers of these species are known to have hypertrophied mandibular glands and release their glandular contents suicidally from the head by rupturing the intersegmental membrane of the gaster. All of the species produce mixtures of polyacetate-derived aromatics, including hydroxyacetophenones, which display pH-dependent color changes, and aliphatic hydrocarbons and alcohols. In addition, three species contained (6*R*)-2,6-dimethyl-(2*E*)-octen-1,8-dioic acid (**9**) or (3*S*)-8-hydroxycitro-nellic acid (**10a**), previously unreported from insects. These compounds were characterized from their spectral data, and confirmed by comparison with synthetic samples. The allomonal role of these compounds

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is based on numerous field observations, and their chemotaxonomic value is presented.

Key Words—*Camponotus* spp., Hymenoptera, Formicidae, polyacetate-derived aromatics, acyclic terpenes, chemotaxonomy, territoriality, autothysis.

INTRODUCTION

Numerous studies of the exocrine chemistry of *Camponotus*, the extremely speciose and geographically widespread genus of carpenter ants (Bolton, 1995), have revealed two patterns: little intrageneric variation in the categories of compounds produced by specific glands, and glandular products with pheromonal, rather than allomonal, roles. For example, recent work has identified branched-skeleton lactones and methylated isocoumarins as products of the *Camponotus* hindgut and straight-chain hydrocarbons and esters from their Dufour's glands and confirmed their roles as trail pheromones and alarm pheromones, respectively (Bestmann et al., 1995, 1997, 1999; Übler et al., 1995; Haak et al., 1996; Kohl et al., 2001, 2003). Earlier investigations of mandibular gland products confirmed aromatic acetogenins such as methyl 6-methylsalicylate (**3**) across species and elucidated the use of these compounds as alarm pheromones and caste-specific flight releasing pheromones (Brand et al., 1973a,b; Lloyd et al., 1984; Blum et al., 1987; Torres et al., 2001). Thus, despite remarkable species proliferation within this genus, exocrine chemistry appears to be quite conserved across geographically and taxonomically diverse *Camponotus* species, and also conserved in its use in communication within, rather than across, species and colonies.

In ants of the *Camponotus cylindricus* complex, workers are unique in exhibiting hypertrophied mandibular glands, extending from the head through the thorax and to the end of the gaster (Maschwitz and Maschwitz, 1974; Buschinger and Maschwitz, 1984). When attacked by predatory ants, and in territorial battles with species inside and outside this group, workers release mandibular gland compounds from the anterior head region by bursting the intersegmental membrane of the gaster (i.e., the abdomen minus its first segment, modified as a petiole or waist; D. W. Davidson and S. C. Cook, unpublished data). Although these workers usually die, their sacrifice may be compensated by the colony retaining its territory intact (Hölldobler and Wilson, 1990). A subset of these species may use the same chemical weapons offensively, a practice that may help to expand the colony's territory (D. W. Davidson and S. C. Cook, unpublished data).

Across these species, the colors of mandibular gland products range from creamy whites to bright white, yellow, orange, and red. They can be seen through the intersegmental membrane of the dorsal gaster when that structure is swollen from feeding, and are displayed in this manner from the upraised gaster when workers are distubed. In a variety of other organisms, such bright colors are

aposematic in function. Identification of the chemical constituents of mandibular gland products should suggest whether potential territorial contestants (and potential predators) have reason to be wary of these exudates.

Here we report a detailed analysis of the whole body extracts of nine *Camponotus* species in the *cylindricus* complex. Among the components of mandibular gland products are several irritants or corrosive irritants, including some phenolic components that exhibit pH-dependent coloration. A number of these aromatic compounds, as well as two acyclic terpene acids, have never before been reported from insects.

METHODS AND MATERIALS

Field Studies. Collections were made at the Kuala Belalong Field Studies Center, run by the Universiti Brunei Darussalam, and located in the Batu Apoi Forest Reserve, Temburong District, Brunei, 4°32′, 115°10′E. Ants from individual colonies were collected separately into absolute methanol and EtOH/water (7:3) in May–July 2001 and 2002, and again in October 2003 (the end of the wet season, and the beginning of a short dry season, respectively). Specimens preserved in ethanol were compared with type material, and vouchers have been deposited in the entomological collections of both the Brunei Museums and the Los Angeles County Natural History Museum. Specific names are available for just three species, *Camponotus cylindricus* (02-85), *C. clerodendri* (02-11), and *C. saundersi* (02-85), and the remaining taxa are identified here by their collection numbers from 2002, and in one case, 2003 (Table 1). Because *cylindricus*-complex species are similar morphologically, the distinctiveness of these collections has been confirmed by amplifying and sequencing cytochrome oxidase subunit I (COI) of mtDNA. A sequence-based phylogenetic treatment of this group will be published elsewhere (S. C. Cook, L. Dickson, and D. W. Davidson, unpublished data). Colors of mandibular gland products were recorded by species during each field season.

Chemical Analysis (U.S.). Gas chromatography–mass spectrometry was carried out in EI mode using a Shimadzu QP-5000 GC–MS equipped with an RTX-5, 30 m × 0.25-mm i.d. column (SGE). The instrument was programmed from 60 to 250°C at 10°C/min. Vapor-phase FT-IR spectra were obtained using an Hewlett-Packard model 5965B detector interfaced with an Hewlett-Packard 5890 gas chromatograph fitted with a 25 m × 0.32 mm HP-5 column. Chiral gas chromatography employed an Hewlett-Packard 5890 instrument using helium as a carrier gas at 22 psi (slightly higher head pressure than normal), equipped with a flame ionization detector and an HP 3390A recorder. The GC was fitted with a 30 m × 0.25-mm i.d. Astec (Whippany, NJ) dimethyl β-cyclodextrin fused-silica column programmed from 70 to 180°C at 0.5°C/min. A permethylated β-cyclodextrin column (SGE), a propionyl γ-cyclodextrin (Astec), and a dialkyl β-cyclodextrin (Astec) column failed to yield useful separations. Isothermal

programs were less successful in separating enantiomers even with the dimethyl β-cyclodextrin column. A head pressure of 20 psi. and a program of 65 to 180°C (0.5°C/min) was used to separate the isomers of **10b**. High resolution mass spectrometry was performed on a JEOL SX102 instrument in the positive-ion fast atom bombardment mode using a direct probe. Ultraviolet spectra were obtained on ethanol solutions using a Shimadzu UV-3100 spectrophotometer. Nuclear magnetic resonance spectrometry was carried out in $CDCl_3$ solutions using a Varian Mercury 400 NMR spectrometer. The aliphatic compounds, phenols, and common terpenes were suggested from their mass spectra (NIST/EPA/NIH. 1999) and retention times and confirmed by comparison with commercial or synthetic authentic samples. 6-Methylsalicylic acid (**3**) and its methyl ester (Hauser and Pogany, 1980), and 2-methyl-5,7-dihydroxychromone (**6**) (Kelly and Kim, 1992) were prepared by published procedures.

Chemical Analyses (Brunei): A number of the chemical studies were replicated in Brunei. There, GC–MS was carried out in the EI mode using an HP 5890 Series II GC - 5971A MSD with HP G1030A MS Chemstation and Wiley 138.L spectra database, and equipped with a Varian CP-Sil 8 CB column (50 m × 0.25-mm i.d., 0.4 microfilm). The instrument was programmed from (a) 60 to 150°C at 2°C/min held for 30 min; then 150 to 200°C at 10°C/min and held for 20 min, or (b) 120°C and held for 20 min then 120 to 200° C at 10°C/min and held for 30 min. Compound identification was confirmed by direct comparison of mass spectra and retention times with those of synthetic or commercial samples.

In collections of three species (02-11, 02-64, and 02-85), a significant component of the gas chromatogram was a broad peak having a mass spectrum, MS m/z (abundance) 182 (3), 164 (20), 149 (2), 136 (25), 95 (71), 69 (14), 67 (26), 55 (43), 45 (48), 43 (75), and 41 (100). Treatment with ethereal diazomethane converted this component into a dimethyl ester, MS m/z 196 (14), 165 (13), 164 (33), 149 (4), 136 (22), 122 (14), 96 (11), 95 (100), 69 (18), 67 (24), 59 (36), 55 (33), 53 (12), 43 (24), and 41 (45). FTIR 2960, 1755, 1735, 1651, 1436, 1359, 1260, 1198, 1163, 1094, and 1015 cm^{-1} HRMS: Calculated for $C_{12}H_{21}O_4$ (M + 1), 229.1440; observed 229.1436. Ozone was bubbled through a small sample of this mixture, followed by a few drops of dimethylsulfide. Analysis of the reaction mixture revealed the presence of methyl pyruvate and a compound whose mass spectrum was similar but not identical to that reported for methyl 7-oxoheptanoate (NIST/EPA/NIH, 1999). Hydrogen was bubbled through a small sample of the dimethyl ester in methanol containing a few milligrams of PtO_2 to provide a single product, MS m/z 199 (2, M-31), 171 (9), 166 (15), 157 (15), 143 (18), 125 (19), 111 (17), 101 (10), 97 (43), 88 (100), 83 (24), 74 (23), 69 (49), 59 (45), 55 (81), 43 (41), 41 (69), identical to that reported for dimethyl 2,6-dimethyloctan-1,8-dioate (Greter et al., 1983).

*Dimethyl (E)-2,6-dimethyl-2-octen-1,8-dioate (**8**) and (E)-2,6-Dimethyl-2-octen-1,8-dioic acid (**9**).* A solution containing 0.3 g of 2-bromopropanoic acid

in 2 ml of dimethoxyethane was slowly added to a solution containing 0.32 g of 60% sodium hydride in mineral oil and 0.3 ml of diethylphosphite in 10 ml of dimethoxyethane under an argon atmosphere. After hydrogen evolution ceased, a solution containing 0.32 g (2 mmol) of methyl 3-methyl-6-oxohexanoate (Yang and Zhang, 2001) in 1 ml of dimethoxyethane was added, and the mixture was stirred at room temperature for 75 min. The usual work up (Brittelli, 1981) followed by reaction with ethereal diazomethane provided 0.35 g (78%) of a 3:2 mixture of dimethylester (**8a**) and an isomeric ethylmethyl ester (**8b**). ^{1}H NMR (400 MHz, $CDCl_3$) **8a**: δ: 6.72 (tq, 1H, $J = 7.6$ Hz, $J = 1.2$ Hz), 3.72 (s, 3H), 3.66 (s, 3H), 2.36–1.92 (m, 4H), 1.82 (s, 3H), 1.5–1.22 (m, 3H), 0.95 (d, 3H, $J = 6.4$ Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 12.59, 19.72, 26.34, 30.29, 35.49, 41.64, 51.70, 51.95, 127.91, 142.25, 168.87, 173.64. The mass spectrum and gas chromatographic retention times of **8a** prepared this way were identical to those of the dimethylester obtained by treating the ant extracts with diazomethane. A sample of this mixture was stirred overnight with LiOH in THF/H_2O. After the THF was removed, the mixture was extracted once with diethyl ether. The aqueous layer was acidified and extracted with ether. Analysis of this extract by GC–MS showed the presence of a single component whose mass spectrum and retention time were identical to those of diacid **9** detected in the ants. Treatment of **9** with ethereal CH_2N_2 provided a pure sample of the dimethyl ester **8a**. The dimethyl β-cyclodextrin column separated the two enantiomers of **8a** prepared from the 3-methyl-6-oxohexanoate intermediate. A chiral sample of **8a** (*S* configuration at C-6) prepared from (3*S*)-methyl citronellate as described above, coeluted with the enantiomer of longer retention time (113.23 min), whereas the natural material from the ant, after methylation with diazomethane, coeluted with the GC peak of shorter retention time (112.76 min) indicating the natural material is of the *R* configuration at C-6. The synthetic (6*S*) diester had 10.4% (6*R*) present, which was shown to come from ca. 10% (3*R*) citronellol (r.t. = 51.29 min) contaminating the sample of (3*S*) citronellol (r.t. = 51.49 min) used to prepare (3*S*)-methyl citronellate.

In collection KB03-10, a significant component in the gas chromatogram was a broad peak having a mass spectrum, MS *m/z* 182 (6), 109 (25), 108 (11), 93 (30), 81 (15), 79 (5), 71 (13), 69 (12), 68 (12), 67 (10), 55 (28), 43 (100), and 41 (70). Treatment with ethereal diazomethane converted this component into a methyl ester, MS *m/z* 182 (3), 165 (13), 151 (2), 122 (10), 109 (18), 108 (25), 107 (4), 93 (53), 81 (15), 69 (11), 68 (9), 67 (10), 59 (20), 55 (30), 43 (100), and 41 (62). FTIR: 3663, 2963, 2928 1757, 1440, 1367, 1291, 1170, 1013, and 841 cm^{-1}. This methyl ester had a GC retention time and mass and FTIR spectra identical with those of a sample of methyl 8-hydroxycitronellate (**10b**) (HRMS: Calculated for $C_{11}H_{21}O_3$ (M + 1), 201.1491; observed 201.1485) prepared from methylcitronellate by catalytic SeO_2 oxidation (Yokoyama and Tsuchikura, 1992; Fairlamb et al., 2001). Methyl 8-acetoxy citronellate (**10c**) prepared from synthetic racemic and (3*S*)-**10b** and from collection KB03-10 (Ac_2O, 2,6-lutidine, 2 d, room

temperature) was used for chiral gas chromatography. The natural and synthetic (3*S*)-**10c** cochromatographed with the tail side of the barely separable racemate (145.25 and 145.35 min for 3*R* and 3*S*, respectively). Coinjection of (3*S*)-**10c** and the natural **10c** gave a sharp peak at 145.5 min indicating they are of the same absolute configuration.

RESULTS

Mandibular gland products from the nine *Camponotus* species included phenols, aliphatics and, in four collections, terpenoids (Table 1). Because the ants were

TABLE 1. COMPOUNDS DETECTED IN *Camponotus* SPP. (*cylindricus* GROUP) COLLECTIONS

Compounds[a]	02-11	02-21	02-52	02-64	02-85	02-100	02-108	02-118	03-10
Phenolics									
m-Cresol (**1**)	14	1			t				1
Resorcinol (**2**)			5			6			
6-Methylsalicylic acid (**3**)	30								
2,4-Dihydroxyacetophenone (**4**)	3	75	40	4	37+	30	1		63
2,4,6-Trihydroxyaceto-phenone (**5**)				15			2		
2-Methyl-5,7-dihydroxy-chromone (**6**)				1	2		21	24	3
Orcinol				t	t				
Aliphatics									
Undecane	7	t	9	1	2	3	6	6	4
Tridecane	1		1						1
Pentadecane	1	t	2	2					2
Heptadecane	2	2	7	2		5	6	10	3
Tetradecanol	1					t	t	t	2
Hexadecanol	2		10	2		t	t	t	7
Octadecanol		2	20	3		15	10	12	t
Eicosanol						6			
2-Heptanone	t	t			t		2	1	
Methyl-3,5-dioxohexanoate		9					3		
Terpenoids									
Citronellal					2				
Citronellol					1				
Citronellic acid					5				
Isopulegol					1				
(6*R*)-(*E*)-2,6-Dimethyl-2-octen-1,8-dioic acid (**9**)	20			44	8				
(*E*)-8-Hydroxy-3,7-dimethyl-6-octenoic acid (**10**)	1			t					10

[a]Gas chromatographic percentages, including fatty acid methyl esters; t = trace.

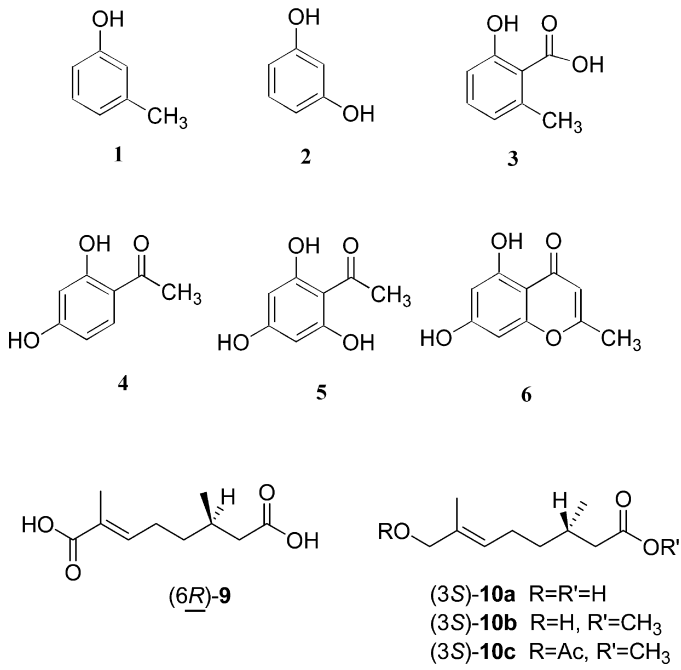


FIG. 1. Major phenolic acetogenins and terpenes from the *Camponotus cylindricus* group.

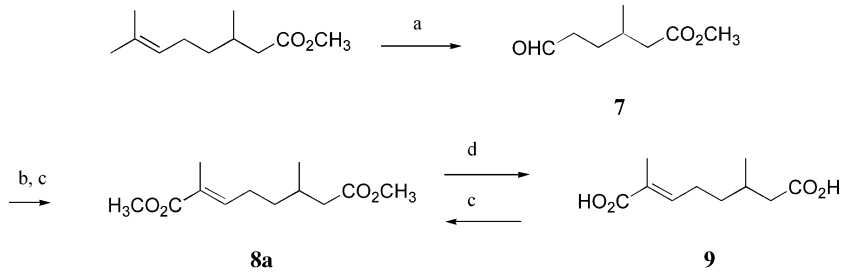
collected into methanol, the usual C-16 and C-18 fatty acids were detected as their methyl esters and are not listed. In addition, small amounts of 3,4-dihydro-3,5-dihydroxy-6-methylpyran-4-one, a common Maillard product previously detected in *Camponotus* (Haak et al., 1996), were found in every species in this investigation. Except for collection 03-10, collected in only 1 year, the qualitative and, for the most part, the quantitative results in Table 1 were consistent over 3 years of collecting.

In *C. cylindricus*, *C. clerodendri*, *C. saundersi*, and in species KB02-64 and KB02-100, mandibular gland products were bright white at the end of the wet season, grading to cream or pale yellow in the dry season and start of the wet season. In collection KB02-52, glandular contents were bright red, grading to peach as conditions dried out and into the start of the wet season. Collection KB03-10 was obtained only in 2003 and exhibited orange secretion at the beginning of the wet season. Both species KB02-118 and species KB02-108 (close or identical to *C.* nr. *saundersi* from Maschwitz and Maschwitz, 1974) consistently produced brilliant yellow products.

All *Camponotus* species in the *cylindricus* complex contained polyacetate-derived aromatics (Figure 1, Table 1), which are likely to be the source of the

bright colors observed in the field. The phenolic ketones **4–6** are known to have pH-dependent ultraviolet spectra (Scott, 1964). For example, the ultraviolet spectrum of **4** showed $\lambda_{max} = 213$ nm ($\varepsilon = 8600$), 231 nm ($\varepsilon = 4700$), and 276 nm ($\varepsilon = 9500$). When the solution was treated with dilute ammonium hydroxide, the absorption at 276 nm shifted to 334 nm ($\varepsilon = 21,000$) in accord with reported shifts (Scott, 1964). The ultraviolet spectrum of **6** exhibited $\lambda_{max} = 227$ nm ($\varepsilon = 6900$), 256 nm ($\varepsilon = 10,000$), and 294 nm ($\varepsilon = 3800$). When the solution was treated with dilute ammonium hydroxide, the absorption at 294 nm shifted to a very broad absorption $\lambda_{max} = 334$ nm ($\varepsilon = 4900$). In a qualitative experiment, the color of a dilute solution of **4** in 1:4 ethanol/water was observed to range from yellow at pH 5.6 to dark pink at pH 7.8.

In *C. cylindricus*, *C. clerodendri*, and species KB02-64, a poorly eluting compound was shown to be a C-10 diacid by virtue of forming a dimethyl ester, $C_{12}H_{21}O_4$, upon treatment with diazomethane. The GC–FTIR spectrum of this diester suggested the presence of a saturated and an unsaturated ester group. Microhydrogenation of the diester gave a product with a mass spectrum identical to that of dimethyl 2,6-dimethyloctan-1,8-dioate, and microozonolysis of the diester gave methylpyruvate as one of the products. These data suggested that the diester was dimethyl 2,6-dimethyl-2-octen-1,8-dioate (**8**), and that the corresponding acid, 2,6-dimethyl-2-octen-1,8-dioic acid (**9**) was the natural compound. This was confirmed by synthesis from methyl citronellate (Scheme 1). The *in situ* Horner–Emmons reaction in this route produces predominantly *E* unsaturated esters (Brittelli, 1981) along with a small amount of the *Z* isomer, allowing the assignment of the natural double bond stereochemistry as *E* by direct comparison of the diacid and diester. Finally, the absolute configuration of **9** was shown to be (6*R*)-2,6-dimethyl-(2*E*)-octen-1,8-dioic acid by GC retention time comparison with the (6*S*)-dimethyl ester on a dimethyl-β-cyclodextrin chiral column. The presence of 8-hydroxycitronellic acid (**10**) in collections KB02-11, KB02-64, and KB03-10 was confirmed by direct comparison of its methyl ester with a synthetic



SCHEME 1. Synthesis of (*E*)-2,6-dimethyl-2-octen-1,8-dioic acid (**9**): (a) $RuCl_3/NaIO_4$; (b) $(EtO)_2PO$, $CH_3CHBrCO_2H$, NaH, then 3-methyl-6-oxohexanoate; (c) CH_2N_2; (d) LiOH.

sample of methyl 8-hydroxycitronellate (**10b**). The absolute configuration of **10** as the 3*S* enantiomer in collection KB03-10 was established using chiral gas chromatography by direct comparison of retention times of 8-acetoxy methyl ester **10c** with synthetic racemic and (3*S*)-**10c**.

DISCUSSION

Chemistry. The chemistry of the *Camponotus* spp. *cylindricus* complex is remarkable for the presence of a variety of polyacetate-derived aromatic compounds along with, in three cases, two acyclic monoterpenes previously unreported in insects, (6*R*)-2,6-dimethyl-(2*E*)-octen-1,8-dioic acid (**9**) and (3*S*)-8-hydroxycitronellic acid (**10a**). While *m*-cresol (**1**) and resorcinol (**2**) are clearly corrosive and irritating compounds, 6-methylsalicylic acid (**3**) as its methyl ester has been reported to be a trail pheromone in other ants (Morgan et al., 1990). The bright colors of the ants and their secretions are likely due to the presence of the phenolic ketones **4**–**6**, because the pH-dependant color changes in 2,4-dihydroxyacetophenone (**4**), 2,4,6-trihydroxyacetophenone (**5**), and 2-methyl-5,7-dihydroxychromone(**6**) are well documented (Scott, 1964). These changes were observed to occur for **4** and **6** over a pH range of 5.8 to 7.8. Because the absorptions for **5** and **6** are at longer wavelengths than **4**, these compounds would be expected to appear even more red as the pH is raised. These compounds might be responsible for the hypothesized "aposematism" in these species (Maschewitz and Maschwitz, 1974; D. W. Davidson and S. C. Cook, unpublished data).

Along with small amounts of common monoterpenes, *C. cylindricus, C. clerodendri*, and species KB02-64 contained (6*R*)-(2*E*)-2,6-dimethyl-octen-1, 8-dioic acid (**9**). In addition, species KB03-10 contained (3*S*)-8-hydroxycitronellic acid (**10a**) (Singh et al., 2000), which was a minor component in *C. clerodendri,* and species KB02-64, although its absolute configuration could not be determined in the latter two species. Whereas **9** and **10** have not been reported previously in insects, **9** is well documented as a urinary metabolite of geraniol and farnesol in mammals (Kuhn et al., 1936; Vaidya et al., 1998), and overproduction of this compound and its congeners can result in toxic acidosis in various species (Bostedor et al., 1997). Diacid **9** may be a dietary metabolite in these ants, one of which has been observed to consume lichen thalli (D. W. Davidson and S. C. Cook, unpublished data). In all three taxa from which this compound is reported, mandibular gland products are white, a color associated with low pH. Diacid **9** may play a role in lowering the pH of the secretion, and seasonal variation in its inclusion in the diet could be involved in the observed seasonal color changes from white to yellow. The differing absolute configurations of **9** and **10** suggest either different dietary sources, or *de novo* biosynthesis by the ants. Indeed, acyclic terpenes such as farnesal, 2,6,10-trimethyl-2,6-undecadiene, and nerolic acid have been reported

in *Crematogaster* species, and nerolic acid has been shown to be a trail pheromone in one species (Haak et al., 1996; Kohl et al., 2003). The terpene acids **9** and **10a** may also play a similar role.

Whereas whole body extracts of burst ants do not permit a precise assignment of a glandular source of all the compounds in Table 1, in analogy with previous work with this genus, the aromatic compounds are most likely the mandibular gland products, and the alkanes and alkanols are from the Dufour's gland. The terpenes may be from the hindgut or Dufour's gland. Nevertheless, chemical signatures of the eight species, not including KB03-10 (collected only once), were consistent across seasons and over 3 years, suggesting that even if the chemical mixtures listed in Table 1 are metabolites, they are species-specific and, therefore, useful as taxonomic indicators. The biosynthesis of phenolic acetogenins such as mellien in *Crematogaster* species has been demonstrated in ants (Bestmann et al., 1997).

Chemical Defenses and Territorial Defense. Extremely territorial weaver ants, *Oecophylla smaragdina* (Hölldobler, 1983), are common components of the undisturbed forest at Kuala Belalong (but see Floren and Linsenmair, 2000), where they have been seen stalking and attacking *Camponotus* sp. KB02-118 (D. W. Davidson, S. C. Cook, and R. R. Snelling, unpublished data) at territorial boundaries. These attacks could represent territorial defense, attempted predation or both. Workers of *Camponotus* species in the *cylindricus* complex burst their defensive glands during confrontations with both weaver ants and one another (though not in several exceptional species pairs), and their aposematic compounds are displayed from a posture (dorsal upraised gaster) that is ideal for threatening other ants in head-to-head competition. On the basis of numerous observations of both natural (unmanipulated) territorial wars, and experiments in which ants were introduced into foreign colonies, it has become certain that the products of hypertrophied mandibular glands are utilized in this context (D. W. Davidson and S. C. Cook, unpublished data).

Effects of the irritant and corrosive irritant aromatic compounds are likely magnified by the adhesives mixed with these compounds. These sticky substances (aliphatics such as octadecanol and perhaps unidentified sugars) adhere the burst *Camponotus* workers to their targets and mire the mandibles of additional enemy ants as these arrive to rescue their nestmates. The sacrifices of *Camponotus* workers are likely justified by the preservation of the foraging territory of the colony as a whole (Hölldobler and Wilson, 1990).

Descended from aculeate wasps (Hölldobler and Wilson, 1990), the earliest ants used stings as offensive and defensive weaponry. Although individual workers were thus capable of engaging the attention of much larger (e.g., vertebrate) enemies, stings were almost certainly less effective in territorial battles with other eusocial insects, including other ants (Buschinger and Maschwitz, 1984; Davidson

et al., 1988). As ants diversified and became one another's most important enemies, nitrogen-limited taxa with herbivorous diets (Davidson, 1997) adopted the use of volatile nitrogen-free chemical sprays (e.g., formic acid, terpenes, ketones, etc.) with which one worker may repel multiple enemies from a colony. Although *Camponotus* species in the *cylindricus* complex appear to have reversed the trend toward volatile sprays, individual workers are still capable of engaging multiple enemy workers with their adhesive irritants.

Chemical Defense Against Predation. *Camponotus* species in the c*ylindricus* complex are unique among members of the Formicinae in deploying persistent, noxious compounds as well as volatile formic acid as defensive compounds. (Formic acid was detected when aspirating the ants; see also Maschwitz and Maschwitz, 1974.) Large-bodied workers in the Dolichoderinae (*Dolichoderus* spp.), the sister group of Formicinae (Baroni-Urbani et al., 1992), are defended by toxins, including iridoid monoterpenes (Cavill and Hinterberger, 1960). However, these species forage in large, long-lived, and conspicuous worker aggregations as specialized tenders of sap-feeding insects (mainly Hemiptera). Under such circumstances, the harvesting of one toxic worker by a naïve predator could have immediate, positive value to the colony as a whole because an experienced predator would be discouraged from feeding on other colony members. In contrast, consumption of solitary-foraging workers of *Camponotus cylindricus*-complex species (D. W. Davidson and S. C. Cook, unpublished data) may provide no such benefit to surviving nestmates.

Thus, the display posture characterizing a subset of *Camponotus* species in the *cylindricus* complex could have evolved in part under selection to deter visually hunting predators such as birds and lizards, but two factors suggest that the ants may be threatened mainly by other arboreal arthropod predators. First, the studied species are all remarkably sensitive to even the slightest leaf vibration (D. Davidson, personal observation). Second, the extraordinary stickiness of the mandibular gland products of all species appears to be integral to the effectiveness of these products, and is apt to be more effective in ensnaring the trophic appendages of arthropods (including weaver ants and spiders) than in deterring vertebrates (see also Maschwitz and Maschwitz, 1974).

Chemistry and Diversification. Our limited ($N = 26$) collections of these ants at Kuala Belalong included a total of nine species, suggesting that this putative clade could be highly diverse and represent recent and rapid proliferation of ecologically equivalent species, with little condensation of species richness to date. Acquisition of persistent toxins and aposematic coloration could have opened a new adaptive zone, wherein workers can forage diurnally and conspicuously on leaf surfaces for dispersed or low density resources, and perhaps with little regard for potential predators. Moreover, once colonies become established in their comparatively long-lived (live) nest trees, their foraging territories may remain

inviolate to incursions by both interspecific and intraspecific competitors, because all species in this complex have similar and similarly effective defenses for maintaining their territories. Molecular phylogenetic studies of this complex of species are currently in progress and may provide additional insight into evolutionary diversification within this group.

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MALE-PRODUCED AGGREGATION PHEROMONE OF THE CERAMBYCID BEETLE *Neoclytus acuminatus acuminatus*

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Abstract—This is the first fully verified report of an aggregation pheromone produced by a cerambycid beetle species. Field bioassays with adult *Neoclytus acuminatus acuminatus* (F.) (Coleoptera: Cerambycidae) revealed that males produce a pheromone that attracts both sexes. Extracts of odors from males contained a single major male-specific compound, (2*S*,3*S*)-hexanediol. Field trials determined that both sexes were attracted by the racemic blend of (2*S*,3*S*)- and (2*R*,3*R*)-hexanediols and that activity was similar to enantiomerically enriched (2*S*,3*S*)-hexanediol (e.e. 80.2%). However, the blend of all four 2,3-hexanediol stereoisomers attracted few beetles, indicating inhibition by one or both of the (2*R**,3*S**)-stereoisomers. Females of the cerambycid *Curius dentatus* Newman were attracted to traps baited with the four component blend, suggesting that a male-produced sex pheromone for this species may contain (2*R*,3*S*)-hexanediol and/or (2*S*,3*R*)-hexanediol. The pheromone of *N. a. acuminatus*, and presumed pheromone of *C. dentatus*, bear structural similarities to those produced by males of six other species in the Cerambycinae (straight chains of 6, 8, or 10 carbons with hydroxyl or carbonyl groups at C_2 and C_3). It is likely that males of other species in this large subfamily produce pheromones that are variations on this structural motif.

Key Words—SPME, red-headed ash borer, *Neoclytus*, *Curius dentatus*, sex pheromone, (2*S*,3*S*)-hexanediol, wood-borer.

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INTRODUCTION

The red-headed ash borer, *Neoclytus acuminatus acuminatus* (F.) (Coleoptera: Cerambycidae, Cerambycinae, tribe Clytini), is endemic to North America, where it infests a variety of hardwood tree species (Linsley, 1964). It also was accidentally introduced into the Adriatic region of southern Europe, where it continues to expand its range (Bense, 1995). The larvae develop in woody tissues of stressed, moribund, and dead trees and can degrade hardwood lumber (Linsley, 1964; Solomon, 1995). Adult *N. a. acuminatus* are present from spring through late summer and are most active between ~11:00 and 17:00 hr (authors' personal observation). They commonly aggregate on larval hosts in groups of more than 40 individuals, and females begin ovipositing immediately after mating (ESL, personal observation). Adults apparently do not feed, and usually live for fewer than 16 d (ESL, personal observation).

During observations of adult *N. a. acuminatus* on the larval hosts in the field and caged beetles in the laboratory, we observed a behavior performed only by males. They would periodically stop walking, fully extend their legs and elevate their body above the substrate, and remain motionless in this position for as long as 30 min. To our knowledge, this behavior has not been reported for any other cerambycid species, and it provided the impetus to investigate the pheromone chemistry of this species. We report here the identification of (2*S*,3*S*)-hexanediol as a male-produced aggregation pheromone for *N. a. acuminatus*.

METHODS AND MATERIALS

Testing for Attractant Pheromones. To test for attractant pheromones in *N. a. acuminatus*, we conducted a field cage bioassay in a ~28 ha arboretum on the campus of the University of Illinois at Urbana—Champaign (UIUC; Champaign Co.). Bioassays were conducted from 13:00 to 16:30 hr on 25 and 26 June 2002; skies clear, air temperatures ~29°C, wind speeds ~8–16 kph. We set three aluminum screen cages (60 × 60 × 20 cm) on the ground, 2 m apart, and 20 m upwind from a pile of ~50 freshly cut logs of green ash (*Fraxinus pennsylvanica* Marshall) that initially harbored at least 40 adult beetles per day. Each cage contained four bolts (~40 × 12 cm) of freshly cut hackberry (*Celtis occidentalis* L.), a natural host of *N. a. acuminatus* (Linsley and Chemsak, 1997). One cage also contained six adult male *N. a. acuminatus*, a second cage contained six adult females, and the last contained only hackberry bolts. We switched positions of cages every hour so that each treatment occupied all positions at least once per day. Beetles used as baits had been captured on larval host material at a different site (Champaign Co., IL) and, prior to the experiment, sexes were housed separately in aluminum screen cages under laboratory conditions (~24°C and ~12L:12D) and provided water. Only beetles that were active and apparently healthy were used. For each

trial, we visually monitored field cages and recorded the number of adult *N. a. acuminatus* landing on cages. These beetles were retained but not sexed. Numbers of beetles responding to cage treatments were compared with a χ^2 goodness-of-fit test (Sokal and Rohlf, 1995).

In an independent bioassay, we used a horizontal glass Y-tube olfactometer (6 cm diam, main tube 26 cm long, arm length 22 cm, 70° angle between arms). We conducted bioassays outdoors because beetles were either sedentary or appeared agitated in the olfactometer under laboratory conditions. Bioassays were preformed in partial shade between 13:00 and 17:00 hr on 14, 15, and 18 May 2003; skies clear, air temperatures ~30°C. We attached a 2 l plastic chamber containing a cylinder of aluminum screen for a perch to each arm of the Y-tube. One chamber held either six males or six females (field captured, as described above) whereas the other was empty. We pulled air through the olfactometer (~2.5 l/min) with a 1.0 hp vacuum cleaner connected to a variable power supply. A beetle was released at the base of the Y-tube and responded to an odor source by crossing a line 18 cm down an arm within 10 min. We bioassayed 20 individuals of each sex for a response to the opposite sex and alternated chambers between Y-tube arms every three trials to control for any positional bias. Numbers of beetles responding to treatments were compared with a χ^2 goodness-of-fit test.

Identification of Pheromone. To identify attractants, we sampled volatile compounds produced by adult *N. a. acuminatus* by both solid phase microextraction (SPME) and by collection of headspace volatiles on an adsorbent. Adults had been reared indoors from infested *F. pennsylvanica* logs, caged as described above for 4–8 d postemergence, and were active and apparently healthy. To sample volatiles by SPME, we placed 10 males and 10 females in separate clean Erlenmeyer flasks, sealed the flasks with corks, and left them on a north-facing laboratory windowsill from 13:00 to 18:00 hr to allow volatiles to accumulate in the flasks. Headspace volatiles were sampled by inserting the SPME fiber (100 μm polydimethylsiloxane, Supelco®, Cat. No. 57300-U, Bellefonte, PA) through the cork and exposing it for 15 min. We desorbed the fiber in the injection port of a Hewlett-Packard® (HP) 5973 mass spectrometer interfaced to an HP 6890 gas chromatograph, using an HP-5MS column (30 m × 0.25 mm × 0.25 μm film) in splitless mode with helium carrier gas. The injection port temperature was 250°C, and the oven temperature was programmed from 40°C for 1 min, 10°C/min to 300°C, and held for 5 min. We sampled volatiles three times from each sex on 17–19 May 2003.

To collect headspace volatiles, we placed 20 adult beetles of the same sex in a glass vacuum trap (~0.3 l) lined with aluminum screen as a perch. A disposable glass pipette containing 100 mg of 80/100 mesh SuperQ® (Alltech Associates, Deerfield, IL) was attached to one nipple of the chamber with a 2-cm long section of Tygon® tubing, and charcoal-purified air was pulled through the apparatus with a vacuum pump at a rate of ~1 l/min. Beetles were aerated on the windowsill

(see above) from 12:00 to 16:00 hr with males sampled on 24 June and females on 25 June 2003. The SuperQ® collectors were eluted with three 0.5-ml aliquots of methylene chloride, and the resulting extracts were analyzed by GC–MS as described above. An initial absence of chromatogram peaks was attributed to volatiles adhering to glass, and the problem was resolved by sonicating vials for 5 min and withdrawing a sample for injection during sonication. The extracts were reanalyzed at the University of California, Riverside, using an HP 6890 GC interfaced to an HP 5973 mass selective detector. The GC was fitted with a DB5-MS column (30 m × 0.25mm, programmed from 40°C/1 min, 10°C/min to 250°C), and injections were made in splitless mode.

The absolute configuration of the insect-produced 2,3-hexanediol was determined by analysis with an HP 5890 GC fitted with a chiral stationary phase Cyclodex B column (30 m × 0.25 mm ID, J&W Scientific), programmed from 50°C/0 min, 5°C/min to 150°C. Under these conditions, a racemic standard was resolved to baseline ($\alpha = 1.015$) with the (2*S*,3*S*)-enantiomer eluting first. The absolute configuration of the insect-produced compound was confirmed by coinjection of an aliquot of the insect extract spiked with the racemic compound.

Synthesis of Pheromone. A solution of OsO_4 (2.5% in *t*-butanol, 5 ml, 0.5 mmol; Aldrich Chemical Co.) was added to an ice-bath cooled mixture of *N*-methylmorpholine oxide (18.9 g, 140 mmol) and (*E*)-2-hexene (12.6 g, 150 mmol; GFS Chemicals, Columbus, OH) in 200 ml of a 3:1 mixture of THF and water. The mixture was warmed to room temperature and stirred for 2 d. The mixture was quenched by addition of 160 ml of a 1 M solution of $Na_2S_2O_5$ in water and stirring for 3 hr. The resulting mixture was extracted ×3 with EtOAc, and the combined extracts were backwashed with 20 ml 3M H_2SO_4 and brine. The solution was dried over anhydrous Na_2SO_4, concentrated, and Kugelrohr distilled at 2 mm Hg (oven temp. ~60–70°C), yielding 5.84 g of racemic (2*R**, 3*R**)-hexanediol as a colorless viscous oil (33%; 96% pure by GC). (2*R**, 3*S**)-Hexanediol was prepared in analogous fashion by oxidation of (*Z*)-2-hexene (GFS Chemical Co.). Spectral data agreed with those previously reported (Schröder et al., 1994).

AD-Mix α (1.4 g, Aldrich Chemical Co.) and methanesulphonamide (95 mg) were dissolved in a two-phase mixture of water (5 ml) and *t*-butanol (5 ml) (Kolb et al., 1994). The mixture was chilled in an ice-bath, and (*E*)-2-hexene (0.125 ml, 1 mmol) was added. The resulting mixture was transferred to a cold room (4°C) and stirred for 44 hr, then quenched by addition of sodium metabisulfite (1.5 g) to the cooled solution, stirred for 30 min, then extracted twice with EtOAc. The combined extracts were washed with brine, dried over anhydrous Na_2SO_4, concentrated, and purified by vacuum flash chromatography on silica gel, eluting with 50% EtOAc in hexanes, yielding enantiomerically enriched (2*S*,3*S*)-hexanediol (e.e. 80.2%, determined on Cyclodex-B column, see above) quantitatively. The (2*R*,3*R*)-enantiomer was made in analogous fashion by using AD-Mix β (Aldrich Chem. Co.) and otherwise identical reaction conditions (e.e. 90.0%).

Field Bioassays of Synthetic Pheromone. We conducted a preliminary field bioassay of racemic (2*R**, 3*R**)-hexanediol in an area adjacent to the UIUC arboretum and in proximity to a pile of freshly cut wood containing sectioned trunks and main branches of several tree species. Logs of white ash (*Fraxinus americana* L.) harbored a population of at least 30 adult *N. a. acuminatus* during the bioassay. We stapled sticky card traps (20.3 × 27.9 cm , Pherocon® AM, Trécé® Inc., Adair, OK) to 1.5 m tall wooden stakes, with the middle of the card ~1 m above the ground. A pair of cards was positioned 2 m apart at distances of 3 and 12 m upwind of the ash logs ($N = 4$ cards). One card of each pair was baited with a rubber septum (10 × 20 mm, Wheaton Science Products, Millville, NJ) loaded with 10 μl of a ~260 mg/ml solution of a racemic blend of (2*R*,3*R*)- and (2*S*,3*S*)-hexanediol in EtOAc clipped to the top of the card, and the other card was baited with a control septum loaded only with EtOAc. We monitored cards for captured beetles continuously because beetles could eventually free themselves. Captured beetles were removed, and the number and sex responding to treatments were recorded. We conducted the bioassay between 14:00 and 17:00 hr on 19 and 20 July 2003; skies clear, air temperature ~27–30°C, wind speed ~8–20 kph. Traps and lures were replaced on the second day. We compared total numbers of beetles responding to the treatment and control with the χ^2 goodness-of-fit test.

To determine the optimum dose of synthetic pheromone for capturing adult *N. a. acuminatus*, we conducted bioassays at four sites. Three sites were within the UIUC arboretum and were separated by at least 200 m, and the remaining site was in Trelease Woods, a ~25 ha mixed hardwood forest, ~11 km NE of the UIUC campus. We expected adult *N. a. acuminatus* would be present at these sites because there was a mature stand of green ash in the arboretum and a variety of host tree species in Trelease Woods. There were no large point sources of beetles, such as recently fallen trees, at any site that could have resulted in positional effects in trap catches. We used "mouse glue trap" cards (Model M180, 12.6 × 22 cm, Victor® Pest Control Products, Lititz, PA) from which the beetles could not escape. Cards were mounted on wooden stakes as described above. At each site we positioned eight stakes 10 m apart in a straight line and approximately perpendicular to the prevailing wind with the sticky surface of cards facing downwind. The pheromone release device was a cotton dental wick (Patterson Dental Supply, Inc., St. Paul, MN) stuck to the card center. The bioassay included eight dosage treatments randomly assigned to traps at each site: 0, 4, 16, 64, 250, 1000, 4000, and 16000 μg of a racemic mixture of (2*S*,3*S*)- and (2*R*,3*R*)-hexanediol in 500 μl of EtOAc. The bioassay was replicated 13 times between 26 July and 18 August 2003; daily air temperatures ~18–35°C, variable cloud cover, negligible precipitation. For each replication, traps were set up at 14:00 hr, and captured beetles were counted, sexed, and removed from cards after 4, 22, 26, 42, and 46 hr. Differences between numbers of females and males captured were tested with the χ^2 goodness-of-fit test. Differences between treatments in

numbers of beetles captured were tested with the nonparametric Kruskal–Wallis test (PROC NPAR1WAY; SAS Institute, 2001). To test differences between pairs of treatment means, we compared 95% confidence intervals (absence of overlap of intervals is a conservative test of statistical significance; Payton et al., 2003). Replications that captured one or no beetles were excluded from the statistical analysis (four of 13 replications).

To determine whether stereoisomeric mixtures were as effective as enantiomerically enriched (2*S*,3*S*)-hexanediol in capturing *N. a. acuminatus*, we conducted field bioassays with an experimental design identical to the previous bioassay, and at the same field sites, but with four traps positioned 15 m apart in each replication. Treatments were loaded on cotton dental wicks and included a solvent control (500 μl of EtOAc), (2*S*,3*S*)-hexanediol of 80.2% e.e., a racemic blend of (2*S*,3*S*)- and (2*R*,3*R*)-hexanediol, and a blend of equal parts of all four 2,3-hexanediol stereoisomers. We based our dosages on the lowest effective dose in the previous study (2000 μg of (2*S*,3*S*)-hexanediol per treatment dose, see Results). The bioassay was replicated nine times between 23 and 28 August 2003; daily temperatures 22–35°C with variable cloud cover, negligible precipitation. Data were analyzed as described above. One replication that captured no beetles was excluded from the analysis.

To determine the effective longevity of pheromone lures, we pooled the data from the two latter field bioassays and compared numbers of beetles captured on the first, second, and third days with the χ^2 goodness-of-fit-test.

Voucher specimens of cerambycid species have been submitted to the Insect Collection of the Illinois Natural History Survey (Champaign, IL).

RESULTS

Testing for an Attractant Pheromone. In the initial bioassay, 32 adult *N. a. acuminatus* arrived on cages containing live males and hackberry logs compared to only five beetles arriving on cages with females and logs, and two beetles on cages with only logs (totals significantly different, $\chi^2_{2,39} = 42.0$, $P < 0.001$). These findings confirmed that males produced an attractant pheromone. During the bioassay, we also observed caged males displaying the characteristic "calling" behavior described in the Introduction.

In Y-olfactometer bioassays conducted outdoors, 16 females responded to odors emitted by males compared to only two responding to the control ($\chi^2_{1,18} = 10.9$, $P < 0.001$). Males showed no significant response to females (7 and 6 males responding to females vs. control, respectively, $\chi^2_{1,13} = 0.08$, $P > 0.05$). In addition, during bioassays using males as the odor source, we captured three wild male and four wild female *N. a. acuminatus* that were attracted to the exhaust vent of the olfactometer vacuum cleaner, indicating that males released a pheromone that was attractive to both sexes.

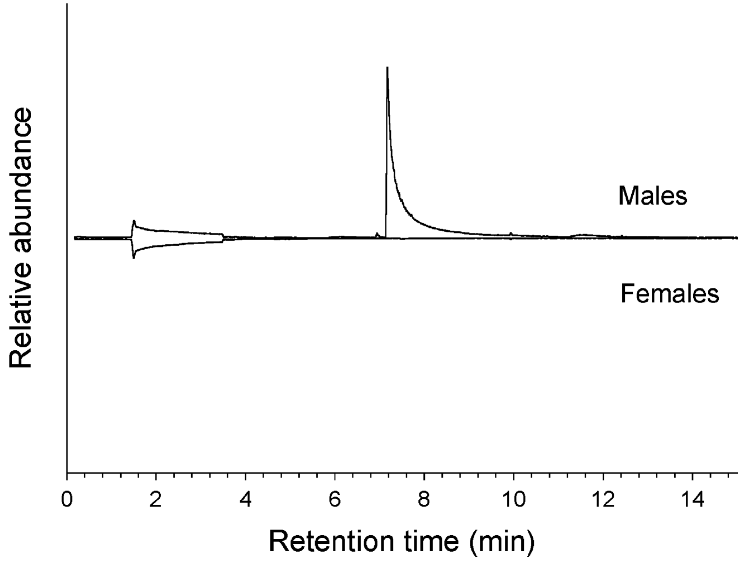

FIG. 1. Representative total ion chromatograms of SPME samples (HP-5MS GC column) of head-space volatiles produced by adult male (top trace) and female (bottom, inverted trace) *N. a. acuminatus*.

Identification of Pheromone. GC–MS analysis of SPME-trapped headspace volatiles from males revealed a single major peak in the total ion chromatogram that was absent in analogous headspace extracts from females (Figure 1). The rather simple mass spectrum had a base peak at m/z 55, and significant fragments at m/z 73 (61), 45 (36), 43 (35), 72 (34), 75 (16), and 57 (20). The highest mass fragment observed was at m/z 103 (1). Although a search of the NIH-NIST computerized mass spectral database gave 4,5-octanediol as the best match, the retention time of an authentic standard was considerably longer than that of the insect-produced compound. The shorter retention time of the insect-produced compound and the similarities of its mass spectrum to that of 4,5-octanediol suggested that the natural compound might be a 2,3-hexanediol. This was confirmed by synthesis of authentic standards. Specifically, the (2R^*,3R^*)- and (2R^*,3S^*)-diastereomers were completely separated on a DB-5MS column, and the insect-produced compound coeluted with the earlier eluting (2R^*,3R^*) enantiomeric pair. The absolute configuration of the insect-produced compound was then determined by analysis of standards and the insect extract on a chiral phase Cyclodex-B column. The insect-produced compound coeluted with the earlier-eluting (2S,3S)-enantiomer on this column, with no trace of the opposite enantiomer. The fact that this compound

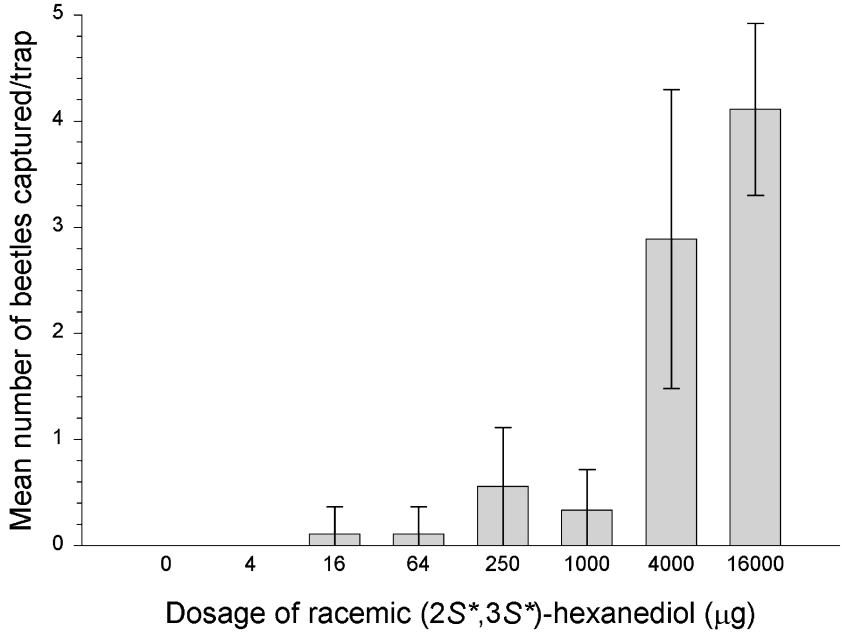

FIG. 2. Relationship between the mean number of adult *N. a. acuminatus* captured on sticky cards (sexes combined) over a 2-d period and dosage of a racemic blend of (2*S*,3*S*)- and (2*R*,3*R*)-hexanediols. Error bars represent 95% confidence intervals ($N = 73$).

was the only compound detected in both SPME and Super Q-collected volatiles from males suggested that the pheromone consisted of a single component.

Field Bioassays of Synthetic Pheromone. Traps baited with the racemic blend of (2*R*,3*R*)- and (2*S*,3*S*)-hexanediols captured six female and six male *N. a. acuminatus* whereas no beetles were captured on control traps (sexes combined, $\chi^2_{1,12} = 12.0$, $P = 0.001$). Beetles responded rapidly to baited traps, with the first beetle arriving within 5 min.

In a subsequent test for optimal dosage, traps captured 42 female and 31 male *N. a. acuminatus* (totals for sexes not different, $\chi^2_{1,73} = 1.66$, $P > 0.05$). There was a significant dose–response relationship (Figure 2; sexes combined, Kruskal–Wallis $\chi^2_{7,73} = 50.2$, $P < 0.001$), with the control and the lowest dosage treatments attracting no beetles, dosages between 16 and 1000 μg attracting < 0.6 beetles/trap (not different from control as indicated by overlap of 95% CI with 0), and greater numbers of beetles (>2.8 beetles/trap) captured with dosages of 4000 and 16000 μg.

In bioassays to test the efficacy of the enantiomerically enriched (2*S*,3*S*)-enantiomer compared to other blends of the stereoisomers, greater numbers of

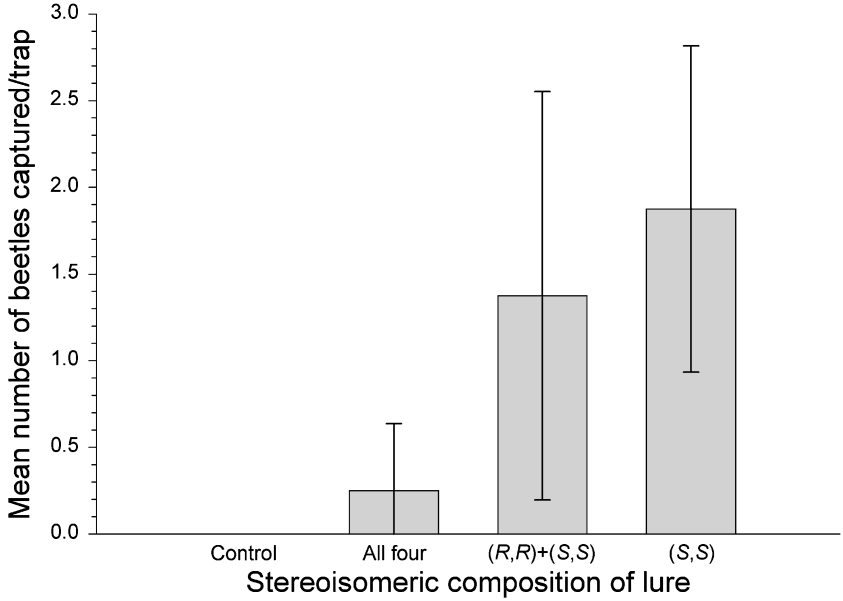

FIG. 3. Relationship between the mean number of adult *N. a. acuminatus* captured on sticky traps (sexes combined) and stereoisomeric composition of the lure: solvent control, blend of equal parts of all four 2,3-hexanediol stereoisomers, racemic blend of (2*S*,3*S*)- and (2*R*,3*R*)-hexanediol, and 80.2% e.e. (2*S*,3*S*)-hexanediol. Error bars represent 95% confidence intervals ($N = 28$).

females were captured on traps than males (20 vs. 8, respectively; $\chi^2_{1,28} = 5.14$, $P < 0.05$). Pheromone treatments differed in average numbers of beetles captured per trap (Figure 3; Kruskal–Wallis $\chi^2_{3,28} = 17.4$, $P < 0.001$), with control traps capturing no beetles, the blend of four stereoisomers attracting numbers of beetles that were no different from the controls (95% CI overlapping with 0), and greater numbers of beetles responding to the racemic blend of (2*S*,3*S*)- and (2*R*,3*R*)-hexanediols and enantiomerically enriched (2*S*,3*S*)-hexanediol (means for the latter two treatments not different). Furthermore, 14 adult females of another cerambycid species, *Curius dentatus* Newman, were attracted to the blend of four stereoisomers, whereas only one was attracted to the enantiomerically enriched (2*S*,3*S*)-hexanediol, and none were attracted to the racemic (2*R**,3*S**) blend or the solvent control (totals different; $\chi^2_{3,15} = 37.5$, $P < 0.001$).

Pheromone traps in both of the latter field bioassays captured totals of 80, 21, and 0 beetles on the first, second, and third days, respectively (totals different, $\chi^2_{2,101} = 102$, $P < 0.001$). These data suggest that the cotton wick release devices were most effective on the first day, and became depleted by evaporation thereafter.

However, the possibility remains that reduced trap captures over time were due to local depletion of the beetle population due to trapping.

DISCUSSION

Both males and females of many cerambycid species are attracted to host plants by their odors, and once on the host, males locate and recognize females by contact chemoreception (Hanks, 1999; Ginzel and Hanks, 2003; Ginzel et al., 2003a,b). Nevertheless, behavioral observations and laboratory bioassays have suggested that volatile pheromones also play a role in mate location in cerambycid species of at least three subfamilies: Prioninae (Edwards, 1961; Benham and Farrar, 1976; Gwynne and Hostetler, 1978), Cerambycinae (Itami and Craig, 1989; Fauziah et al., 1992), and Lamiinae (Wang et al., 1991; Kim et al., 1992). The putative attractants have yet to be identified for any of these species. More detailed studies of pheromone chemistry using electroantennography, laboratory bioassays, and/or field bioassays have been carried out with another seven cerambycid species in two subfamilies. For these species, pheromone components have been identified, and their activity has been confirmed in some (Table 1). Pheromones were produced by males in all of these species.

Within the subfamily Lamiinae, male-specific volatile compounds have been identified only for *Anoplophora glabripennis* Motsch. (Table 1), and both sexes responded to the synthetic compounds in olfactometer bioassays (Zhang et al., 2002). Male-specific compounds identified from the six species of the Cerambycinae (tribes Anaglyptini, Callidiini, and Clytini) are structurally similar and composed of one to three compounds that have hydroxyl or carbonyl groups at C_2 and C_3 of 6, 8, or 10 carbon chains (Table 1). The biological activity of these compounds as attractant pheromones has been confirmed for four of these species, suggesting that similar compounds in the remaining two are also pheromones. The male-produced pheromone, (2*S*,3*S*)-hexanediol, that attracted both sexes of *N. a. acuminatus* in our study, fits this general pattern of pheromone production (Table 1). The structures of these compounds are entirely different from the female-produced pheromones of species in families closely related to the cerambycids. For example, N-(2′*S*)-methylbutanoyl-2-methylbutylamine is a pheromone component for *Migdolus fryanus* Westwood (Coleoptera: Anoplodermatidae, sensu Svacha and Danilevsky, 1987; Bento et al., 1992, 1993; Leal et al., 1994), and (*S*)-10-oxoisopiperitenone has been identified as a pheromone of *Vesperus xatarti* Dufour (Coleoptera: Vesperidae; Boyer et al., 1997).

Whereas the equivalent activity of enantiomerically enriched (2*S*,3*S*)-hexanediol and the (2*R**,3*R**) racemic blend suggests that (2*R*,3*R*)-hexanediol is not inhibitory to *N. a. acuminatus*, the negligible attractiveness of the blend of four stereoisomers indicates that one or both of the (2*R**,3*S**) stereoisomers

TABLE 1. SUMMARY OF PUBLISHED RESEARCH ON ATTRACTANT PHEROMONES OF MALE CERAMBYCIDAE AND BIOASSAYS USED TO CONFIRM ACTIVITY

Subfamily (Tribe)	Species	Pheromone components	Activity of candidate pheromone	References
Lamiinae (Monochamini)	*Anoplophora glabripennis* Motsch.	4-(*n*-heptyloxy)butanal, 4-(*n*-heptyloxy)butan-1-ol	Antennae of both sexes respond; both sexes attracted in lab bioassays	Zhang et al., 2002
Cerambycinae (Anaglyptini)	*Anaglyptus subfasciatus* Pic	(3*R*)-hydroxy-2-hexanone; (3*R*)-hydroxy-2-octanone	♀ weakly attracted in field bioassays, response of ♂ not significant	Nakamuta et al., 1994, 1997; Leal et al., 1995
Cerambycinae (Callidiini)	*Hylotrupes bajulus* (L.)	(3*R*)-hydroxy-2-hexanone; (2*R*,3*R*)- and (2*S*,3*R*)-hexanediol	♀ attracted in lab bioassays; response of ♂ not tested	Schröder et al., 1994; Fettköther et al., 1995
Cerambycinae (Callidiini)	*Pyrrhidium sanguineum* (L.)	(3*R*)-hydroxy-2-hexanone; (2*R*,3*R*)- and (2*S*,3*R*)-hexanediol	Activity not tested	Schröder et al., 1994
Cerambycinae (Clytini)	*Xylotrechus pyrrhoderus* Bates	(2*S*,3*S*)-octanediol; (2*S*)-hydroxy-3-octanone	Antennae of both sexes respond; ♀ attracted in lab bioassays	Iwabuchi, 1982, 1988; Sakai et al., 1984; Iwabuchi et al., 1985, 1986
Cerambycinae (Clytini)	*Xylotrechus chinensis* Chevrolat	2,3-octanediol; 2-hydroxy-3-octanone, 3-hydroxy-2-octanone	Activity not tested	Iwabuchi et al., 1987; Kuwahara et al., 1987
Cerambycinae (Clytini)	*Xylotrechus quadripes* Chevrolat	(2*S*)-hydroxy-3-decanone	Antennae of both sexes respond; ♀ weakly attracted in lab bioassays, ♂ not attracted; weak or no response by either sex in field bioassays	Hall et al.,1998; Rhainds et al., 2001

inhibits attraction. Our serendipitous capture of female but not male *Curius dentatus* (Cerambycinae, tribe Curiini), in traps baited with the mixture of the four 2,3-hexanediol stereoisomers but not with (2*S*,3*S*)-hexanediol or the (2*R**,3*R**) racemic blend, indicated that (2*R*,3*S*)- and/or (2*S*,3*R*)-hexanediol probably constitute a male-produced sex pheromone of this species. With this evidence that species representing four tribes of the Cerambycinae use C_6 to C_{10} vicinal diol or hydroxyketone pheromones, it seems likely that males of other species in the large subfamily Cerambycinae also produce pheromones with this structural motif.

To our knowledge, this is the first fully verified report of an aggregation pheromone in the Cerambycidae. Male-produced aggregation pheromones have been identified in many insect orders (Schlyter and Birgersson, 1999), and in at least nine families of the Coleoptera, including several species in the Chrysomelidae (Rao et al., 2003), the group most closely related to the Cerambycidae (Farrell, 1998), and in other types of wood-borers, including the Bostrichidae, Platypodidae, and Scolytidae (Schlyter and Birgersson, 1999). Response of males to male-produced pheromones may be adaptive if multiple males are more effective in attracting females and acquiring mates than are individual males, although the mechanisms underlying evolution of group calling in insects remain controversial (see Shelly and Whittier, 1997).

There is some evidence of intrasexual activity of male-produced pheromones in other species of the Cerambycinae (see Table 1). For example, male-produced pheromones stimulated antennae of both sexes of *Xylotrechus pyrrhoderus* and *X. quadripes* (Iwabuchi et al., 1985; Rhainds et al., 2001). Pheromones produced by male *Anaglyptus subfasciatus* attracted males as well as females in field bioassays (Nakamuta et al., 1997), whereas further research is needed to determine the full spectrum of activity of male-produced pheromones in *Hylotrupes bajulus, Pyrrhidium sanguineum, Xylotrechus pyrrhoderus*, and *X. chinensis* (see references in Table 1). Taken together, the fragmented body of literature summarized in Table 1 emphasizes the gaps in our knowledge of the role of pheromones in mate location in cerambycid beetles.

Although we captured fewer than five beetles/trap during 2-d bioassays, pheromone traps that have been tested for other cerambycid species have been even less effective, capturing only a few beetles over periods of several weeks to months (e.g., Nakamuta et al., 1997; Rhainds et al., 2001). Moreover, our field bioassays revealed that adult *N. a. acuminatus* responded to pheromones over distances of at least 100 m, if not greater distances, because there was no source of adult beetles in proximity to traps in some of our field trials. In contrast, female *X. pyrrhoderus* were reported to respond to pheromones of males only over distances of less than 2 m (Iwabuchi, 1982). Furthermore, it is possible that attraction of *N. a. acuminatus* to traps could be enhanced by combining host plant volatiles with pheromones, as has been demonstrated with *A. subfasciatus* (Nakamuta et al., 1997).

Curius dentatus is a little-known species that previously had not been reported from east central Illinois, where we conducted our studies (see Linsley, 1963; Yanega, 1996), as evidenced by the absence of specimens from Illinois in extensive collections of the Field Museum of Natural History (Chicago, IL) and the Illinois Natural History Survey (Champaign, IL; personal observations). Our capture of this species demonstrates that pheromone trapping can be a sensitive method for detecting cerambycid species. Thus, cerambycid pheromones may prove to be valuable in developing detection and quarantine programs for invasive exotic cerambycid species. For example, the pheromone of *N. a. acuminatus* has already been used in a pheromone trap-based survey for this species in New Zealand, where it recently was introduced accidentally, and where it may well become a serious pest (D. M. Suckling, HortResearch, Lincoln, NZ, personal communication).

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INVESTIGATION OF LONG-RANGE FEMALE SEX PHEROMONE OF THE EUROPEAN TARNISHED PLANT BUG, *Lygus rugulipennis*: CHEMICAL, ELECTROPHYSIOLOGICAL, AND FIELD STUDIES

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Abstract—The European tarnished plant bug, *Lygus rugulipennis*, is an important pest of agricultural and horticultural crops throughout Europe. Adult male *L. rugulipennis* were previously shown to be attracted to traps baited with live virgin females, which suggests the females produce a sex pheromone. Volatiles produced by virgin female *L. rugulipennis* were shown to contain three components, hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal which elicited electroantennographic (EAG) responses from males in analyses by linked gas chromatography–electroantennography (GC-EAG). They were produced in 1.5:1:0.08 ratio, respectively, by single females. Collections from 1, 2, or 4 virgin females showed the proportions of hexyl butyrate and (*E*)-4-oxo-2-hexenal to increase relative to that of (*E*)-2-hexenyl butyrate with increasing number of females. Although these compounds were found in body extracts of both male and female *L. rugulipennis*, they were not detected in volatiles released by virgin males. EAG dose–response studies showed that both males and females responded to these chemicals with minimal differences in sensitivity between the sexes or to the three components, except that males were more responsive than females to (*E*)-4-oxo-2-hexenal at the two highest doses tested. Release rates of the compounds from rubber septa, polyethylene vials, and polyethylene sachets were measured under laboratory conditions. Four field tests were carried out using sticky traps baited with all possible binary and

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tertiary combinations of the three chemicals using different combinations of dispensing systems. Catches of male *L. rugulipennis* in baited traps were similar to those in unbaited traps. Significantly fewer females were caught on traps baited with blends containing hexyl butyrate than on traps without hexyl butyrate or unbaited traps in one test and overall. The roles of the three compounds and possible reasons for their failure to attract males are discussed.

Key Words—*Lygus rugulipennis*, Heteroptera, Miridae, tarnished plant bug, pheromone, electroantennography, repellence, hexyl butyrate, (*E*)-2-hexenyl butyrate, (*E*)-4-oxo-2-hexenal.

INTRODUCTION

The European tarnished plant bug, *Lygus rugulipennis Poppius* (Heteroptera: Miridae), is an important pest of agricultural and horticultural crops throughout Europe, including lucerne (Erdelyi et al., 1994), wheat (Holopainen, 1989), and strawberries (Taksdal and Sorum, 1971). It has been recorded on over 320 host plants in 57 families (Holopainen, 1989). On strawberries, adults and nymphs feed in the flowers, resulting in the malformation of fruit (Taksdal and Sorum, 1971; Cross and Easterbrook, unpublished), and significant damage is caused at population densities of less than one bug per 20 plants (Cross, unpublished). Growers previously used short-persistence, broad-spectrum organophosphate insecticides such as malathion or heptenophos in mid- and late summer to control the pest, but these materials have recently been withdrawn from use. As part of a program to develop alternatives and a more integrated approach to management of this pest, improved methods of monitoring are being investigated. Populations of *L. rugulipennis* can be assessed by beating or suction sampling, but these methods are time consuming and are not likely to be used widely by growers. Pheromone-baited traps could provide a practical alternative. Initial studies showed that traps baited with live, virgin female *L. rugulipennis* attracted conspecific males in greenhouses (Innocenzi et al., 1998). This indicated that the females produce a sex pheromone and in a preliminary communication we reported identification of three compounds in volatiles produced by virgin females of this species (Innocenzi et al., 1998). In this paper, we describe details of the identification of these chemicals, comparison of their electroantennographic activity toward male and female *L. rugulipennis*, and field trapping experiments with blends of the chemicals.

METHODS AND MATERIALS

Insect Material. L. rugulipennis were reared at 20°C under a 16L:8D photoperiod in perspex plant propagators (80 × 30 × 40 cm; B&Q, Kent, UK).

A mixture of green beans, *Phaseolus vulgaris*, and strawberry plants, *Fragaria ananassa*, was used as adult food source and oviposition site. Beans and strawberry plants were replaced every 3 d and the material removed was placed in petri dishes sealed with sticky tape. On hatching, nymphs were moved to propagators containing multiple layers of torn-up tissue roll and fresh pieces of green bean which were replaced every 2 d. On emergence, adults were sexed and segregated in plastic boxes (15 × 10 × 8 cm; B&Q, Kent, UK) containing tissue paper and green beans.

Collection of Volatiles. Volatiles were collected from natural or synthetic sources placed in a glass chamber (10 × 3 cm diam). A diaphragm pump (Capex Mk II, Charles Austen, UK) was used to draw air (1 1/min) into the chamber through an activated charcoal filter (20 × 2 cm, 6–18 mesh) and out through a trap containing Porapak Q (200 mg, 50–80 mesh, Waters Corp., MA 01757, USA), held between plugs of silanized glass wool in a Pasteur pipette. The Porapak was purified by Soxhlet extraction with chloroform for 8 hr, and traps were rinsed well with dichloromethane immediately before use. Adsorbed volatiles were removed from the traps with dichloromethane (pesticide grade; 1 × 0.5 ml, 1 × 1 ml). To collect insect-produced volatiles, one to four virgin male or female *L. rugulipennis* adults (6–10-d-old) from groups as above were placed in the chambers on strawberry flowers or green beans for 24–72 hr at 20°C with a 16L:8D cycle. Collections were analyzed by linked gas chromatography–electroantennography (GC-EAG) and gas chromatography–mass spectrometry (GC-MS) as below. Amounts of components present were estimated by comparison of peak areas with those of synthetic standards.

Extraction of Body Chemicals. Body chemicals were extracted from individual males or females (3–15-d-old) between 7 and 9 hr into the photophase, after they had been subjected to air-entrainment for 24 hr. The bug was chilled in a refrigerator (4°C), the head and wings removed and the remaining thorax and abdomen placed in dichloromethane (100 μl). The sample was filtered through glass wool, undecane (10 μg) was added as internal standard, and the mixture was stored at −20°C until analysis.

Gas Chromatography (GC). GC analyses were carried out using Carlo Erba Mega 5300 instruments equipped with fused silica capillary columns (25 m × 0.32 mm i.d.) coated with polar CPWax52CB (Carbowax 20M equivalent; Chrompack, London, UK) or nonpolar CPSil5CB (methyl silicone; Chrompack). The carrier gas was helium at 50 kPa, and the oven temperature was held at 50°C for 2 min then programmed at 6°C/min to 240°C. Injector and detector temperatures were 200 and 240°C, respectively. Injection was splitless and detection was by flame ionization detection (FID). Data were captured and processed using EzChrom 6.1 software (Aston Scientific, Stoke-Manderville, Bucks, UK). Retention indices (RI) were calculated relative to the retention times of straight-chain hydrocarbons.

Gas Chromatography–Mass Spectrometry (GC-MS). GC-MS analyses were carried out with a Carlo Erba Mega 5300 GC linked directly to a Finnigan ITD 700 ion trap detector (Thermoquest, Hemel Hempstead, Herts, UK) operated in electron impact (EI) or chemical ionization (CI) with isobutane mode. GC columns and conditions were as above except that column i.d. was 0.25 mm. Spectra were compared with those in the NBS/NIH/EPA library, published spectra, and a library generated from samples analyzed previously at NRI.

Electroantennography (EAG). As described by Cork et al. (1990), after brief anaesthetization with carbon dioxide, the whole insect was placed on a plasticine block on its ventral surface, covered with a strip of filter paper and its antennae restrained with fine copper wire staples. Glass capillaries (50 mm $\times$ 0.2 mm diam) containing 0.5 M aqueous KCl solution with 1% polyvinylpyrrolidine to reduce evaporation were placed over silver wire electrodes attached to a DC amplifier (UN06, Syntech, Hilversum, The Netherlands). The electrodes were held in place by micromanipulators (Leitz, Wetzlab, Germany). The indifferent electrode was placed in the connective tissue between the scape and pedicel of one antenna and the recording electrode in the connecting tissue of the two flagella segments of the other antenna. The amplifier was connected to a PE Nelson 5300 interface, and the output captured and processed using Turbochrom 4 software (Perkin–Elmer–Nelson, Beaconsfield, Bucks, UK).

For linked GC-EAG analyses, virgin males (5–13-d-old) were used. GC columns and conditions were as above, and effluent from the GC outlet was split equally between the FID and the insect preparation. The GC column effluent was blown at 15-sec intervals over the EAG preparation with a 3-sec pulse of nitrogen (500 ml/min) (Cork et al., 1990). GC data were also recorded and processed using Turbochrom 4 software.

For EAG dose–response studies, the preparation was exposed to charcoal-filtered air (1 l/min.) through a glass Y-piece (5 mm i.d.) positioned 1 cm away from the antenna. A glass Pasteur pipette containing the chemical stimulus was positioned in the remaining Y-piece arm. The test solution (5 μl) was placed on a piece of filter paper (Whatman no. 1, 1 cm^2) in the glass Pasteur pipette. The pipette was attached to tubing leading to a nitrogen source preprogrammed to deliver a 3-sec pulse of nitrogen (1 l/min). Immediately before attaching the pipette, the tubing was purged with a pulse of nitrogen. Virgin adult *L. rugulipennis* (5–13-d-old) were used, and the insect was subjected to three pulses of nitrogen at 1-min intervals for each of the test solutions. The solutions were administered in increasing concentrations, but different test compounds of the same concentration were randomly assigned. An interval of 2–3 min was allowed between concentration sets. Dichloromethane (5 μl) was pulsed over the preparation at the beginning and end of every concentration set, and a standard, (*E*)-2-hexenal (5 μg), was pulsed before and after every three test chemical deliveries. The EAG response was taken as the maximum deflection from the baseline

as measured during the nitrogen pulse. The response amplitude was calibrated using a digital voltmeter (1044 Voltage and Current Calibrator, Time Electronics, Tonbridge, UK).

For analysis, the magnitudes of the second and third responses to the stimulus were added together and averaged. This was then transformed to a percentage of the average of the two standard responses on either side of the odorant. The response to the first pulse was disregarded as results showed that this response varied little between the three chemicals, suggesting it was principally due to the solvent. Absolute responses to account for solvent and other background effects were obtained by subtracting the averaged EAG response to solvent recorded before and after each concentration set (Dickens, 1984).

Chemicals. Butyrate esters were synthesized by reaction of butyryl chloride with the corresponding alcohol in dichloromethane in the presence of pyridine at 0°C followed by aqueous workup and distillation: hexyl butyrate (98% purity, b.p. 76°C/7 mm), (*E*)-2-hexenyl butyrate (99% purity, b.p. 120°C/10 mm). (*E*)-4-Oxo-2-hexenal (99% purity, b.p. 100°C/5 mm) was synthesized according to Ward and van Dorp (1969) or by a route similar to that subsequently described by Marques et al. (2000)

Dispensers. Dispensers tested included white rubber septa (20 mm × 10 mm o.d.; Sigma–Aldrich, Gillingham, Dorset, UK, cat. no. Z10,072-2), polyethylene vials (26 mm × 8 mm × 1.5 mm thick; Just Plastics Ltd., London) and polyethylene sachets (25 mm × 25 mm × 120 μm thick) prepared by heat sealing (Adion Elektro, The Netherlands) white, translucent lay-flat tubing (International Pheromone Systems Ltd., South Wirral, UK). Rubber septa were impregnated with hexyl butyrate (5 mg) by applying a solution in petroleum spirit (b.p. 40–60°C, 0.1 ml) and allowing the solvent to evaporate in a fume hood at room temperature. In laboratory and field studies, polyethylene vials and sachets were loaded with hexyl butyrate, (*E*)-2-hexenyl butyrate, or a blend of the two in 3:2 ratio (100 mg), or (*E*)-4-oxo-2-hexenal (20–40 mg). For measurement of release rates, duplicate samples of dispensers were maintained in a laboratory wind tunnel (27°C, 8 kph windspeed) with periodic weighing and trapping of volatiles emitted over 1 hr at 27°C as described above. Volatile collections from mixtures were analyzed by GC using undecane or octyl acetate (5 μg) as internal standard. Residual pheromone in rubber septa exposed in the wind tunnel for different intervals was extracted overnight at room temperature with hexane (5 ml) containing octyl acetate (1 mg) as internal standard and quantified by GC analysis.

Field Trials. Field experiments were done at HRI, East Malling, and, unless otherwise stated, were as follows. Traps were made from wooden stakes (60 cm) with two white, square plastic sheets coated with polybutene adhesive (20 × 20 cm; Oecos, Kimpton, Herts., UK), attached vertically one above the other at a relative angle of 90°. A pink hair-roller (Tesco Supermarkets, UK), capped at both ends with white, sterilin tube lids was centered on top of the wooden stake and

secured with an upholstery nail. The dispenser was placed inside the hair-roller. Latin Square and randomized complete block experimental designs were used and the spacing between traps was 15 m × 10 m. Treatments were unbaited (treatment A), hexyl butyrate + (*E*)-2-hexenyl butyrate (treatment B), hexyl butyrate + (*E*)-4-oxo-2-hexenal (treatment C), (*E*)-2-hexenyl butyrate + (*E*)-4-oxo-2-hexenal (treatment D), hexyl butyrate + (*E*)-2-hexenyl butyrate + (*E*)-4-oxo-2-hexenal (treatment E). Traps were examined and the number of insects recorded at least once a week. Lures were renewed every 2 wk.

In 1998, one experiment was done in an abandoned 2-year-old Everbearer strawberry plot. Each chemical was dispensed from an individual polyethylene vial. The trial was run from 14 August to 28 September 1998 (5 × 5 Latin Square), and trap catches were recorded weekly.

In 1999, three experiments were done in purpose-sown fields of weeds including mayweed, *Matricaria recutita*, fathen, *Chenopodium album*, and groundsel, *Senecio vulgaris*, which are all known hosts of *L. rugulipennis*. For the first experiment from 23 July to 18 August (5 × 5 Latin Square) lures were a polyethylene sachet for (*E*)-4-oxo-2-hexenal and separate polyethylene vials for the two butyrates. For the second experiment from 19 August to 26 September (5 × 5 Latin Square) the sachet and vial dispensers were replaced with a single vial for all treatments. The third experiment was in an adjacent weed field, separated by a windbreak composed of *Alnus cordata* and was run from 8 August to 9 September 1999 (randomized complete block design with three replicates). The two butyrates and (*E*)-4-oxo-2-hexenal were dispensed from individual polyethylene vials as in 1998.

Statistical Analyses. For electrophysiological studies, male and female responses to a specific dose of compound were compared using a two-tailed *t* test. Comparisons of pairs of chemicals within a sex for a specific dose were analyzed using a paired *t* test if analysis of variance (ANOVA) for all three chemicals showed significant differences. Data from the field experiments were analyzed using a general linear model (SPSS v 10 software; SPSS Inc., Chicago, IL 60606, USA) after square root transformation. A significance level of 5% was used for all statistics.

RESULTS

Identification of EAG-Active Chemicals. In GC-EAG analyses of volatiles from virgin female *L. rugulipennis* using a male *L. rugulipennis* EAG preparation, three electrophysiologically active compounds were detected (A, B, and C in Figure 1). The pulsing procedure used in these analyses (Cork et al., 1990) gives a regular, relatively slow response from mechanoreceptors on the antenna. An active chemical in the pulse causes a much more rapid response from the chemoreceptors,

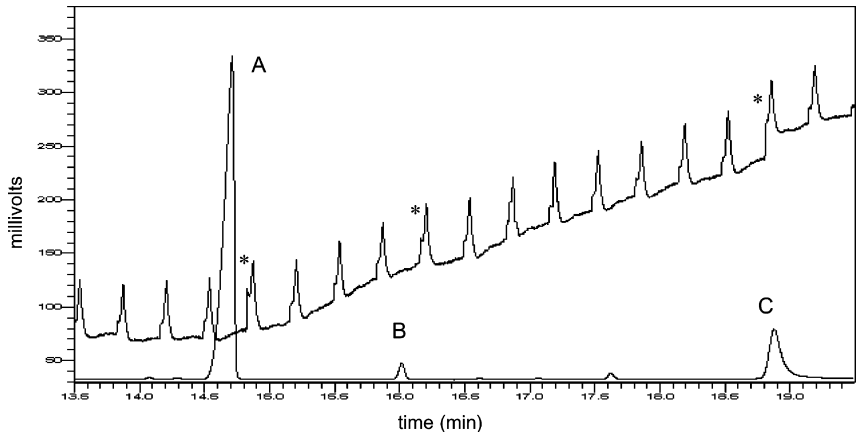

FIG. 1. GC-EAG analysis of volatiles from virgin female *L. rugulipennis* using virgin male EAG preparation (CPWax column; upper trace EAG, lower trace GC; * corresponding EAG response).

seen on the front of the mechanoreceptor response (Figure 1). These compounds were not present in virgin male emissions when these were analyzed by GC-EAG or subsequently by GC-MS (Figure 2). GC retention indices for the active components are shown in Table 1.

Mass spectral analyses of the first two EAG-active components (A) and (B) and comparison with mass spectral library data suggested the compounds were hexyl butyrate and (*E*)-2-hexenyl butyrate, respectively. Identity of (A) was confirmed by comparison of retention indices and cochromatography with synthetic hexyl butyrate on both polar and non-polar GC columns (Table 1) and comparison of the mass spectrum with that of the synthetic compound. Six of the seven hexenyl butyrate isomers were synthesized, and comparison of retention data for these with those of EAG-active component (B) (Table 1) showed the latter were identical only with those for (*E*)-2-hexenyl butyrate. Mass spectra of (*E*)- and (*Z*)-2-hexenyl butyrates were similar, but different from those of the other isomers (Figure 3). The former spectra have a prominent ion at m/z 83 corresponding to $(C_4H_9{-}CH{=}CH)^+$ which is not as stabilized in the other isomers. They also have a base peak at m/z 71 ($C_3H_7{-}CO^+$) and a significant ion at m/z 100, presumably due to the corresponding hexenol half of the molecule. Spectra of the other isomers have abundant ions at m/z 82 due to McClafferty arrangement which is not favored in the 2-isomers as it would involve the olefinic hydrogen and perhaps less favored in the (*Z*)-3-isomer because of steric interference in the transition state by the *Z* double bond.

EAG-active component (C) in the volatiles from female *L. rugulipennis* had an EI mass spectrum dominated by three ions at m/z 55 (100%), 83 (85%), and 112

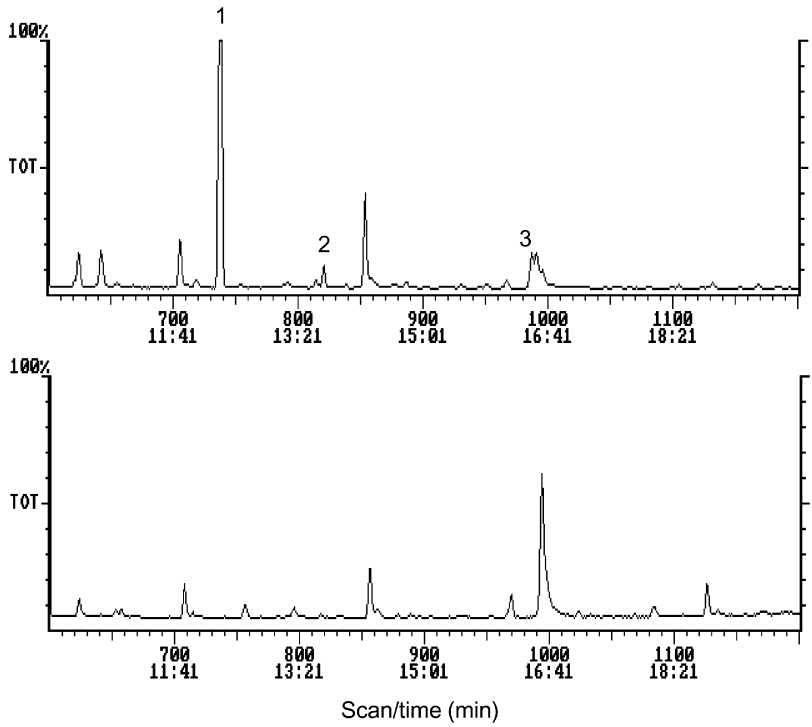

FIG. 2. GC-MS analysis of volatiles from virgin female (upper trace) and virgin male (lower trace) *L. rugulipennis* adults (CPWax column; 1= hexyl butyrate; 2 = (*E*)-2-hexenyl butyrate; 3 = (*E*)-4-oxo-2-hexenal).

(1.2%). The CI mass spectrum showed a strong M + 1 ion at m/z 113, indicating a molecular weight of 112. The marked difference in retention times on polar and non-polar columns (Table 1) suggested a highly polar compound, and (C) was proposed to be (*E*)-4-oxo-2-hexenal, shown previously to be produced by other heteropteran bugs (e.g., Aldrich, 1988). This identification was confirmed by comparison of the GC retention times and mass spectra with those of a synthetic standard.

The relative amounts of the three components produced by virgin female *L. rugulipennis* over seven collections with four individuals in each collection were 35:1:2 hexyl butyrate:(*E*)-2-hexenyl butyrate:(*E*)-4-oxo-2-hexenal, respectively. However, the ratio of components was dependent on the number of individuals entrained such that reducing the number of females increased the amount of (*E*)-2-hexenyl butyrate relative to those of the other two components (Table 2). Accurate quantification was not done, but by comparison of GC peak areas with those of external standards, the emission rate of hexyl butyrate was estimated to be

TABLE 1. RETENTION INDICES (RI) OF EAG-ACTIVE COMPONENTS AND SYNTHETIC COMPOUNDS

Compound	Retention index	
	CP Wax 52CB	CP Sil5CB[a]
EAG active (A)	1419	1175
EAG active (B)	1480	1175
EAG active (C)	1599	838
Hexyl butyrate	1415	1176
(*E*)-2-Hexenyl butyrate	1475	1175
(*Z*)-2-Hexenyl butyrate	1459	1175
(*E*)-3-Hexenyl butyrate	1453	1167
(*Z*)-3-Hexenyl butyrate	1461	1168
(*E*)-4-Hexenyl butyrate	1478	1178
5-Hexenyl butyrate	1489	1183
(*E*)-4-Oxo-2-hexenal	1595	837

[a] Retention data for EAG-active components taken from GC-MS analyses.

approximately 10 μg/d per female for groups of four females and approximately 1 μg/d from individual females. These values equate to 0.42 μg/hr and 0.042 μg/hr, respectively, assuming continuous, uniform release during the collection period.

Analysis of Body Extracts. GC analyses of body extracts showed both male and female *L. rugulipennis* contained all three of the compounds identified in volatiles from the females (Table 3). At 3–5 d, amounts of hexyl butyrate were significantly greater in females than males, but at 10–15 d amounts were significantly greater in males than females. The ratios of the three components were similar in females and males at 3–5 d and females at 10–15 d, and the ratios more closely resembled those observed in volatile collections from groups of four females (Table 2). Body extracts of males at 10–15 d showed proportionately more (*E*)-4-oxo-2-hexenal.

Electroantennogram Dose–Response Studies. Serial dilutions of hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal in dichloromethane were made up within the range 1 ng/μl–100 μg/μl in steps increasing by an order of magnitude. Preliminary work showed no differences in the EAG responses of *L. rugulipennis* to the three most dilute solutions, and the two lowest concentrations were not tested further in order to reduce the time testing each insect and avoid unnecessary fatigue.

In EAG dose–response studies, antennae of both male and female *L. rugulipennis* responded to all three components. Antennae of male *L. rugulipennis* were on average 30% ($N = 20$) more responsive than females to the standard, (*E*)-2-hexenal, but this difference was not significant ($P = 0.061$). In terms of mean absolute EAG responses corrected for response to solvent, males were no

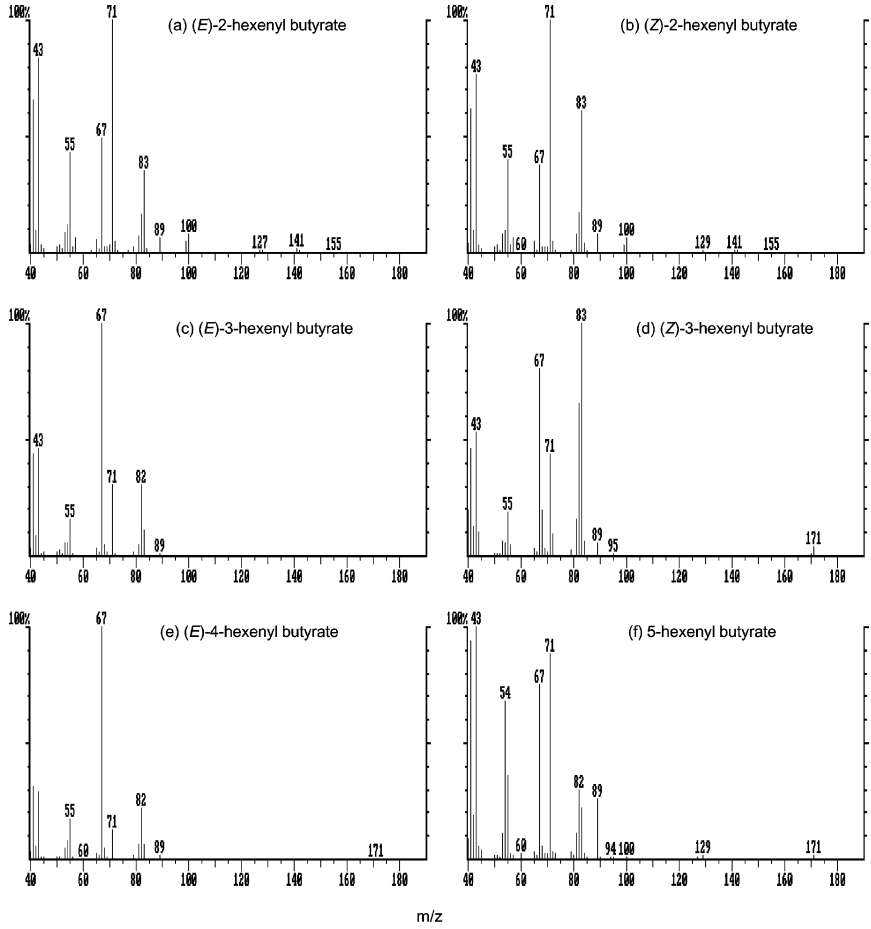

FIG. 3. Mass spectra of positional and geometrical of hexenyl butyrate isomers.

TABLE 2. RELATIVE RATIOS OF HEXYL BUTYRATE, (*E*)-2-HEXENYL BUTYRATE, AND (*E*)-4-OXO-2-HEXENAL PRODUCED BY VIRGIN FEMALE *L. rugulipennis* (AGE 7–21 DAYS) IN GROUPS OF 1–4 INDIVIDUALS

No. entrained	No. replicates	Average age (d)	Hexyl butyrate: (*E*)-2-hexenyl butyrate: (*E*)-4-oxo-2-hexenal
4	7	11	35.00:1.00:2.00
2	5	12	3.00:1.00:0.50
1	17	10	1.50:1.00:0.08

TABLE 3. AMOUNTS OF HEXYL BUTYRATE, (*E*)-2-HEXENYL BUTYRATE AND (*E*)-4-OXO-2-HEXENAL FOUND IN BODY EXTRACTS OF MALE AND FEMALE *L. rugulipennis*[a]

	Female (μg/body)			Male (μg/body)		
Age	Hexyl butyrate	(*E*)-2-Hexenyl butyrate	(*E*)-4-Oxo-2-hexenal	Hexyl butyrate	(*E*)-2-Hexenyl butyrate	(*E*)-4-Oxo-2-hexenal
3–5 d ($n = 3$)	11.61*	0.62	0.42	4.10*	0.23	0.11
(SE)	(2.35)	(0.18)	(0.19)	(1.35)	(0.08)	(0.04)
Ratio	18.7	1.0	0.7	17.8	1.0	0.5
10–15 d ($n = 5$)	14.22*	1.05	0.87**	46.22*	1.71	20.36**
(SE)	(3.18)	(0.56)	(0.65)	(10.67)	(0.58)	(5.51)
Ratio	13.5	1.0	0.8	18.4	1.0	9.5

[a]* or ** indicates a significant difference at the $P < 0.05$ and $P < 0.005$ level, respectively, for female–male comparisons of chemicals in a particular age range.

more responsive than females ($P > 0.05$) except for (*E*)-2-hexenyl butyrate at the 5 μg dose.

For comparison of responses of each sex to the three test compounds. responses were normalized to the response to the standard (*E*)-2-hexenal (5 μg) (Figure 4). Males showed no differences in responses to the three compounds

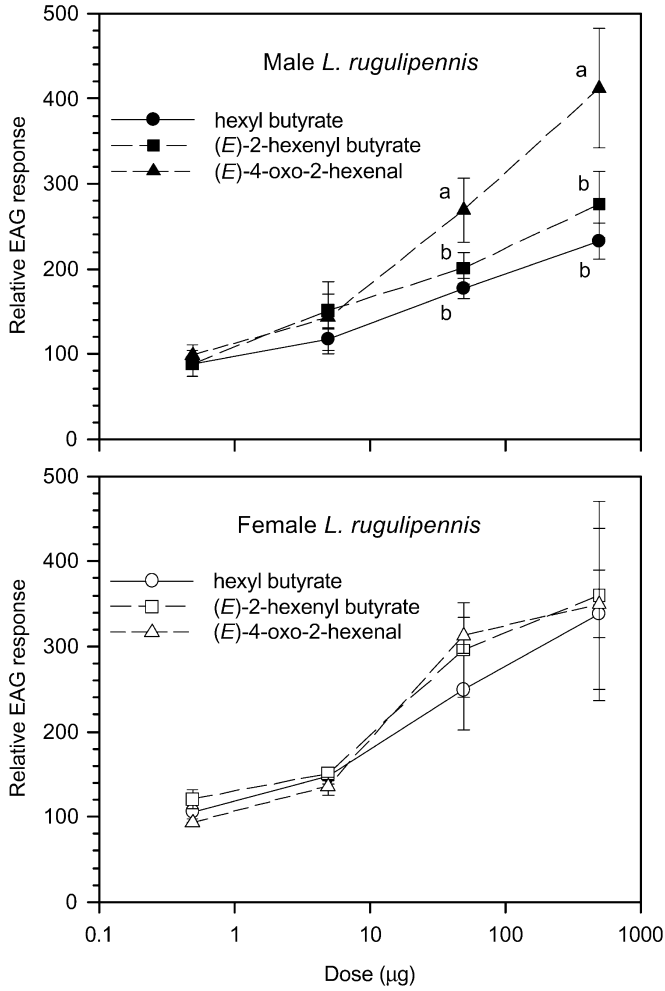

FIG. 4. Normalized EAG responses of antennae of virgin male (upper) and female (lower) *L. rugulipennis* to hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal. (Significant differences ($P < 0.05$) between responses to the different compounds for a particular source dose are indicated by different letters.)

except at the two highest doses, where (*E*)-4-oxo-2-hexenal elicited a greater response than either hexyl butyrate or (*E*)-2-hexenyl butyrate ($P < 0.05$). Female *L. rugulipennis* showed much smaller differences in EAG responses to the three test chemicals (Figure 4), and one-way ANOVA showed no differences ($P > 0.05$) among any of the four doses tested.

Dispenser Studies. Release of hexyl butyrate (5 mg) from rubber septa exposed in a laboratory wind tunnel at 27°C at 8 kmph windspeed was measured by GC quantification of residual material at intervals, and of volatiles released during a 1-hr period. Amounts of hexyl butyrate remaining immediately after evaporation of the solvent and after 1, 2, 3, 4, 7, 9, and 11 d were 71.7, 13.2, 4.7, 1.9, 0.8, 0.14, 0.08, and 0.06% of the initial dose, respectively. Release rates at the same time intervals were 91.2, 14.5, 2.5, 0.9, 0.4, 0.10, and 0.04 μg/hr, respectively. In view of the rapid loss of material and nonuniform release rate, these dispensers were not investigated further.

Release of hexyl butyrate or (*E*)-2-hexenyl butyrate from separate polyethylene sachets exposed in the laboratory wind tunnel was rapid (1.7 and 1.5 mg/hr, respectively) continuing at a constant rate until exhaustion of the contents (cf. Torr et al., 1997). The release rate of (*E*)-4-oxo-2-hexenal from the sachets was constant for the first 15 d at 1.4 mg/hr but then decreased, possibly due to compound decomposition.

Measurement of release of hexyl butyrate or of (*E*)-2-hexenyl butyrate from separate polyethylene vials exposed in the laboratory wind tunnel showed virtually no release for the first 3 d as the chemicals penetrated through the polyethylene. The release rate then increased to constant rates of 0.16 and 0.15 mg/hr, respectively, for at least 17 d. The release rate of (*E*)-4-oxo-2-hexenal was much slower at 0.0075 mg/hr.

Release of compounds from mixtures was determined by weighing to derive total weight loss followed by GC analysis of trapped volatiles to establish relative proportions of each chemical released. Whenever (*E*)-4-oxo-2-hexenal was in the presence of hexyl butyrate, the relative amount of (*E*)-4-oxo-2-hexenal released decreased to zero within 1 wk by GC analysis. This coincided with the appearance of two additional peaks, suggesting chemical transformation. In contrast, the (*E*)-2-hexenyl butyrate and (*E*)-4-oxo-2-hexenal mixture was relatively stable. The release rate of (*E*)-2-hexenyl butyrate from a 3:1 blend was constant at 0.04 mg/hr and that of (*E*)-4-oxo-2-hexenal decreased during the first week from 0.025 mg/hr to a constant rate of approximately 0.01 mg/hr. A mixture of hexyl butyrate and (*E*)-2-hexenyl butyrate (3:2) was released at 0.11 mg/hr, and the ratio of the two components in the volatiles was 2:1, respectively.

Field Trials. Four field trials were carried out to compare catches of male and female *L. rugulipennis* in unbaited traps and traps baited with one of the three binary or the tertiary blend of the three components using different dispensing systems. The results are summarized in Figure 5. Catches of male bugs with

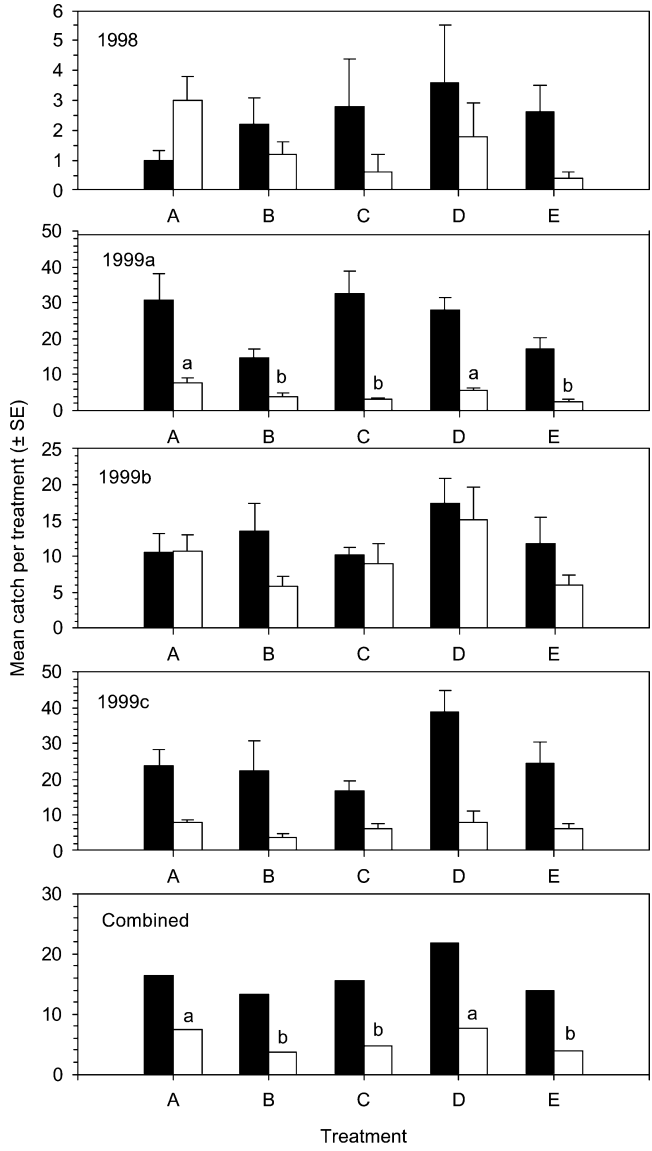

FIG. 5. Mean total trap catches (±SE) of male and female *L. rugulipennis* in four experiments over two seasons. (Males ■, females □; treatments: (A) unbaited; (B) hexyl butyrate + (*E*)-2-hexenyl butyrate; (C) hexyl butyrate + (*E*)-4-oxo-2-hexenal; (D) (*E*)-2-hexenyl butyrate + (*E*)-4-oxo-2-hexenal; (*E*) hexyl butyrate + (*E*)-2-hexenyl butyrate + (*E*)-4-oxo-2-hexenal; means with different letters are significantly different ($P < 0.05$) for that sex in that experiment after square root transformation of the data and ANOVA.)

the blend of (*E*)-2-hexenyl butyrate and (*E*)-4-oxo-2-hexenal (treatment D) were numerically highest in three of the four tests, but none of the differences were significant ($P > 0.05$). Even combining the data from all four trials failed to show any significant differences ($P = 0.27$) after analysis of variance treating each trial as a replicate.

Catches of females in baited traps were generally less than catches of males and mostly less than those in the unbaited traps (Figure 5). The latter differences were significant ($P < 0.05$) in the first trial in 1999 for traps baited with hexyl butyrate and (*E*)-2-hexenyl butyrate (treatment B), hexyl butyrate and (*E*)-4-oxo-2-hexenal (C), or the blend of all three components (E), but not for those baited with the blend of (*E*)-2-hexenyl butyrate and (*E*)-4-oxo-2-hexenal (D). In this trial, (*E*)-4-oxo-2-hexenal was dispensed from a polyethylene sachet giving a higher release rate than in the other trials, whereas hexyl butyrate and (*E*)-2-hexenyl butyrate were dispensed from separate polyethylene vials. A similar pattern was observed on combining results of all the trials, and simple analysis of variance, treating each trial as a replicate, followed by an LSD test indicated numbers caught in traps baited with treatments (B), (C), and (*E*) were lower than those in unbaited traps (A) or traps baited with treatment (D) ($P < 0.05$) (Figure 5).

DISCUSSION

Chemical Studies. Three compounds, hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal were found in whole body extracts of both virgin female and male *L. rugulipennis*. These compounds have previously been found in the metathoracic glands of the related species *L. lineolaris* (Gueldner and Parrot, 1978; Aldrich, 1988), *Lycogoris pabulinus* (Drijfhout et al., 2002), and *L. hesperus* (Ho and Millar, 2002). The amount of hexyl butyrate was significantly higher in males than in females at 10–15 d old, and the amounts of just the three components found here indicate a gland content at least twice that estimated for *L. lineolaris* by Blumenthal (1978).

However, when volatiles were collected from one to four virgin female or male *L. rugulipennis* that had been previously reared in groups, the three compounds were only detected in volatiles collected from females. This is in contrast to results from *L. lineolaris* (Wardle et al., 2003), *Lycogoris pabulinus* (Groot et al., 2001; Drijfhout et al., 2002), and *L. hesperus* (Ho and Millar, 2002) where these compounds were found to be emitted by both sexes. Wardle et al. (2003) were studying these chemicals for their alarm, epidietic, or defensive roles. Even when collecting volatiles from "calm" adults, they used groups of 10 individuals in a vessel approximately half the size of that used here, and amounts collected from males and females were similar. However, Groot et al. (2001) and Drijfhout et al. (2002) used relatively well-separated insects for collection of volatiles (5–7 bugs in a 250 ml container) and Ho and Millar (2002) used similar conditions (1 bug in

20 ml) to those used here for *L. rugulipennis*. In these latter two examples, amounts of compounds collected from males were markedly less than the amounts from females.

Analyses of collections of volatiles from different numbers of *L. rugulipennis* females showed that the absolute amounts and the relative ratios of the chemical compounds released by an individual female depended on the number of females present. Female *L. rugulipennis* produced hexyl butyrate at approximately 40 ng/hr when entrained individually, compared to 400 ng/hr per female when entrained in groups of four. These figures are comparable to the values of 10–92 ng/hr and 25–1081 ng/hr reported for calm and disturbed female *Lycogoris pabulinus,* respectively, by Groot et al. (2001) and the 60–80 ng/hr collected from individual *L. hesperus* by Ho and Millar (2002). Wardle et al. (2003) reported 85 ng/hr and 23,653 ng/hr of hexyl butyrate, respectively, from calm and disturbed female *L. lineolaris* with similar amounts from the males.

In collections of volatiles from female *L. rugulipennis*, the ratio of hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal changed from 1.5:1:0.08 from one female to 35:1:2 for four females. The latter ratio is closer to that found in the body extracts of *L. rugulipennis* females. The changes observed in the absolute and relative amounts of chemicals released by individual female *L. rugulipennis* in solitary and grouped situations may reflect an epideictic function used for density regulation. A defensive or alarm function seems less likely: male *L. rugulipennis* do not produce the compounds, individually or in groups of up to four, comparable to *Lycogoris pabulinus* where both calm and highly disturbed males produce hexyl butyrate at similar very low levels. If the compounds are components of the sex pheromone, increased production by females in groups might be simply a response to intraspecific competition to attract males or could have more complex effects on sexual communication. Thus, Groot et al. (2001) showed high levels of hexyl butyrate reduced the attractiveness of female *Lycogoris pabulinus* to males, probably by inhibiting pheromone production by the females. Zhang and Aldrich (2003a) reported that high levels of hexyl butyrate and (*E*)-2-hexenyl butyrate produced by male *L. lineolaris* interrupted attraction of the males to the female sex pheromone. Such regulation of chemical production appears to be common in the Heteroptera (e.g., Marques et al., 2000) and has the potential to increase the versatility of individual chemicals and mixtures of chemicals in insect communication (Blum, 1996).

Electrophysiological Studies. In linked GC-EAG analyses of volatiles collected from virgin female *L. rugulipennis* using a male EAG preparation, responses were only recorded to the three compounds hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal. A similar result was obtained with *Lycogoris pabulinus* by Drijfhout et al. (2002), and these authors speculated that the compounds may be used in long-range, conspecific communication. Subsequent dose–response studies with the synthetic compounds showed that antennae of both male and female *L. rugulipennis* gave EAG responses to all three compounds. Responses

from males were always higher than those from females, but in all but one case the differences were not significant. Females showed similar responses to the three compounds at all doses tested, whereas males gave significantly higher responses to (*E*)-4-oxo-2-hexenal than to the other two compounds at the two highest doses tested. For *L. lineolaris*, Chinta et al. (1994) found males showed higher EAG responses than females to hexyl butyrate and (*E*)-2-hexenyl butyrate, significantly so at some doses. Responses to (*E*)-4-oxo-2-hexenal were not tested.

Dispenser Studies. Prior to field trials, different dispensing systems for the three chemicals were evaluated in the laboratory. Polyethylene vials and polyethylene sachets were found to give sustained release over at least 2 wk, in contrast to rubber septa which released the majority of material loaded within 24 hr. Unexpectedly, combining hexyl butyrate and (*E*)-4-oxo-2-hexenal in the same dispenser caused significant decomposition of the latter, whereas (*E*)-4-oxo-2-hexenal was relatively stable in combination with (*E*)-2-hexenyl butyrate. (*E*)-4-Oxo-2-hexenal is known to be rather unstable to dimerization (Aldrich et al., 1993) and presumably oxidation (e.g., Marques et al., 2000). Possibly the olefinic double bond in (*E*)-2-hexenyl butyrate provides some sort of radical trap that reduces oxidation and perhaps dimerization.

According to these studies, the dispensing system that most closely simulated the blend produced by a single virgin female *L. rugulipennis* would consist of a polyethylene vial containing a 3:2 blend of hexyl butyrate and (*E*)-2-hexenyl butyrate, with (*E*)-4-oxo-2-hexenal in a separate polyethylene vial. This system produced a relative release ratio of 2:1:0.2 of the three components, respectively, which is a reasonable approximation of the 1.5:1.0:0.08 blend produced by a female *L. rugulipennis*, although it could obviously be refined further. Although the absolute amounts dispensed from the polyethylene vials (approximately 0.15 mg/hr) were more than three orders of magnitude greater than the amounts found to be produced by a single virgin female (40 ng/hr), other mirids are known to respond to high release rates (e.g., McBrien and Millar, 1999).

Field Trapping Studies. The analytical and electrophysiological results described here, taken together with those of Wardle et al. (2003) and Groot et al. (2001) on related species, would be consistent with the proposal that the three compounds hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal could act as components of the female sex pheromone and also have alarm, epidietic, and/or defensive roles depending upon amounts released and the ratios of the components (cf. Blum, 1981, 1996). In greenhouse trapping trials demonstrating attractiveness of virgin female *L. rugulipennis* to males (Innocenzi et al., 1998), traps were baited with three females in a container of similar size to that used for the volatile collections here.

Four field trapping trials were carried out in 1998 and 1999, comparing catches in unbaited traps with those in traps baited with one of the three binary mixtures of hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal or the tertiary mixture, using different combinations of dispensers. Both male and

female *L. rugulipennis* were caught in all the traps, but there were no differences in catches of males between baited and unbaited traps ($P > 0.05$) in any of the individual trials or if the results were combined. Catches of males were generally higher in baited than unbaited traps except for the first trial in 1999 in which hexyl butyrate and (*E*)-2-hexenyl butyrate were dispensed from polyethylene vials and (*E*)-4-oxo-2-hexenal from a polyethylene sachet to give a relatively higher release rate of the latter.

Catches of female *L. rugulipennis* were generally lower than those of males, and mostly lower in baited traps than those in the unbaited traps. In the first trial in 1999 and the combined results of all four trials, catches in traps baited with the binary or tertiary blends containing hexyl butyrate (Figure 5, treatments B, C, and E) were significantly lower ($P < 0.05$) than those in the unbaited traps or traps baited with the binary blend not containing hexyl butyrate (Figure 5, treatment D). The above results would suggest the latter compound is an important component of any alarm or epidietic function for these chemicals, consistent with the still-air laboratory bioassay results of Wardle et al. (2003) on *L. lineolaris*. These authors showed hexyl butyrate alone is repellent to both males and females, and (*E*)-4-oxo-2-hexenal is not necessary for repellency. Although not the objective of the present study this is the first demonstration that these compounds can have a significant repellent effect under field trapping conditions. Despite demonstrating such activity for *L. lineolaris* in laboratory bioassays, Wardle et al. (2003) could not show any effect in field cage studies.

The failure of these field tests to demonstrate any significant attraction of male *L. rugulipennis* is not unprecedented in studies of the pheromones of *Lygus* species (e.g., Hedin et al., 1985; Ho and Millar, 2002) and may be due to one or more factors. The three compounds, hexyl butyrate, (*E*)-2-hexenyl butyrate, and (*E*)-4-oxo-2-hexenal, may not be sufficient or even necessary for attraction. However, the fact that they were released only by virgin female *L. rugulipennis* and not by males suggests that they are involved in the female sex pheromone. They were the only active components detected in GC-EAG analyses of volatiles collected from virgin females using a male EAG preparation, but there is always the possibility that other minor components were missed. Components of alarm and epidietic pheromones would be expected to elicit EAG responses in both sexes and few significant differences were observed in responses of female and male *L. rugulipennis* to the synthetic compounds.

It is possible that relative amounts of the compounds released during the field tests were not suitable, but laboratory measurement of release rates indicated that the dispensing systems used in most of the trials gave a reasonable approximation to the blend produced by a single virgin female *L. rugulipennis*. However, it should be noted that the single components were not tested in the field studies.

Another possible reason for failure of the field trials is that the overall release rate was too high, release rates from the polythylene vials being at least three orders

of magnitude greater than the 40 ng/hr estimated for single female *L. rugulipennis*. However, that the latter figure is probably an underestimate in that it was derived from a value for release over a 24-hr period, whereas it is likely that release of these compounds was not constant or uniform throughout this period. In previous work on trapping with synthetic sex pheromones of other mirid lugs, only Smith et al. (1991) reported dispenser release rates. These ranged from 25 to 183 μg/d (1.0–7.6 μg/hr) which is significantly less than used here. Kakazaki and Sugie (2001) used glass capillary tubes containing microgram quantities of pheromone components to attract males of the mirid bug, *Trigonotylus caelestialium*. Their experiments ran for 2 wk and release rates were not reported, but in our hands these dispensers released the pheromone components at approximately 0.5 μg/hr for 2–3 d at 27°C after which pheromone components were undetectable in analyses of volatiles released or residues in the tubes (unpublished results). It is also worth noting that Millar et al. (1997), Millar and Rice (1998), and Zhang and Aldrich (2003b) used rubber septa to dispense synthetic pheromone components of several mirid species with successful results. In our hands, septa loaded with 5 mg of hexyl butyrate released most of this within 2 d and the release rate fell rapidly from approximately 100 μg/hr to less than 1 μg/hr during this time. However, in contrast to the microcapillary tubes above, detectable amounts continued to be released from the septa for at least 11 d at 27°C.

A final possibility is that it is important to have a single point source for pheromone release in order to attract male *L. rugulipennis*. In the work described here, two or three dispensers were often used because of the different polarity and volatility of (*E*)-4-oxo-2-hexenal compared with those of hexyl butyrate and (*E*)-2-hexenyl butyrate and also because (*E*)-4-oxo-2-hexenal was less stable in the presence of hexyl butyrate. Every effort was made to position the dispensers as close together as possible, but Todd and Baker (1999) have reported that even if this is done the plumes from the different dispensers can remain separate for distances of several metres downwind.

Work is in progress to investigate these possible explanations and continue attempts to demonstrate attraction of *L. rugulipennis* males to synthetic pheromone components in the field.

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IDENTIFICATION OF A SEX PHEROMONE COMPONENT FOR THE BLUEBERRY LEAFMINER, *Caloptilia porphyretica*

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Abstract—Coupled gas chromatographic–electroantennographic detection (GC–EAD) of both gland extracts and effluvial collections from female blueberry leafminer, *Caloptilia porphyretica* Braun (Lepidoptera: Gracillariidae), showed that females produced a single EAD-active compound. The amount of the compound collected from virgin female *C. porphyretica* was below GC and mass spectrometry (MS) detection thresholds, even with highly concentrated gland extracts (~150 female equivalent). (*E*)-11-Hexadecenal (*E*11-16:Ald) was determined to be a sex pheromone component mainly by comparison of retention times with authentic standards on both polar and nonpolar capillary columns, microreaction-GC–EAD analyses, and field trapping tests. GC–EAD experiments showed that synthetic *E*11-16:Ald exhibited extraordinarily high electrophysiological activity, stimulating significant male antennal responses at as low as 10 fg. Traps baited with *E*11-16:Ald alone were attractive to males. Addition of 1 or 3% of its geometric isomer, *Z*11-16:Ald, to *E*11-16:Ald did not significantly increase trap captures, but an inhibitory effect was observed at the 10% level. The influence of two kinds of rubber septa on attraction was also evaluated. Male moth captures were higher in traps baited with red rubber septa than with gray rubber septa at 30–300-μg doses. Monitoring of adult flight activity with 3-μg doses of *E*11-16:Ald indicated at least three distinct flight periods throughout the 2003 season.

Key Words—Blueberry leafminer, *Caloptilia porphyretica*, sex pheromone, (*E*)-11-hexadecenal, gas chromatography–electroantennogram detection, microreactions, population monitoring, field trapping.

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INTRODUCTION

The blueberry leafminer, *Caloptilia porphyretica* Braun, has become a frequent pest of commercial highbush blueberries, *Vaccinium corymbosum* L., grown in Atlantic and Burlington Counties of New Jersey. This insect was first reported to infest azaleas in North Carolina (Braun, 1923). In recent years, severe infestations, with as high as 40–50% damaged leaves, have been observed in many locations in New Jersey.

Adult *C. porphyretica* are small moths with a wing span of ~1.5 cm. Eggs are deposited singly on the abaxial surface of the leaf, and at times more than one egg may be deposited on each leaf. Upon hatching, the larva bores through the lower wall of the egg and the leaf cuticle into the epidermis. The first two instars are sap feeders, whereas the later instars feed on leaf tissue within or outside the mine (Davis, 1987). The fourth instar larvae exit the mine and using silk, fold a single leaf into a symmetrical, triangular tent and feed on leaf tissue while remaining within the tent. Larvae leave the tents when mature and usually pupate on the abaxial leaf surface. This insect overwinters as a mature larva enclosed within a cocoon on senesced leaves on the soil surface. At present, there are no publications available on the biology and seasonal life history of *C. porphyretica*.

Although blueberry bushes can tolerate moderate leaf mining without loss of production, severe infestations may affect the plant vigor and cause yield loss. In addition to direct production loss, this insect can become a contaminant, especially in machine-harvested fruit, as the larvae drop from bushes along with the harvested fruit. The presence of larvae in harvested fruit can substantially increase sorting time on the packing lines and may result in a contaminated product.

The objectives of this study were to identify the sex pheromone of *C. porphyretica*, and to develop a pheromone lure for population monitoring. Sex-pheromone-baited traps will be useful in the study of the seasonal life history of *C. porphyretica*. The identification of the sex pheromone will enable possible future development of mating disruption and attract-and-kill technologies for managing *C. porphyretica* populations.

METHODS AND MATERIALS

Insects. Larvae of *C. porphyretica* were collected from commercial blueberry fields, near Hammonton, Atlantic County, in New Jersey during June–October of 2002. These larvae were individually reared in Chatsworth, NJ, in screen-top plastic containers (10 × 4 cm) in an incubator at 25°C and 16L:8D photoperiod. Moths that emerged were sexed and allowed to mate in a large screened wooden cage (1 × 1 × 1.5 m) with three to four 1-year-old blueberry plants, maintained in

a greenhouse at 20–27°C and 16L:8D photoperiod. Absorbent cotton moistened with 8% sugar water was provided as a food source for moths. Approximately 3 wk later, pupae were collected and kept individually in screen-top plastic containers in an incubator at 20°C and 16L:8D photoperiod until emergence. After emergence, moths were sexed and transferred to 15°C and 16L:8D photoperiod until used in experiments in Beltsville, MD.

Pheromone Gland Extractions. Pheromone gland extracts were obtained during photophase from seven groups of 5- to 20-d-old virgin females that were kept at 15°C and 16L:8D or previously used for effluvial collections (3, 15, 20, 30, 30, 100, and 150 females per group). A female abdomen was compressed gently until the ovipositor everted. The ovipositor was excised with microscissors into a conical glass vial containing ~100 μl methylene chloride/methanol (3:1). The glands were soaked for at least 2 hr at room temperature. Extracts were removed, and the glands were re-extracted with 100 μl methylene chloride/methanol. The combined solution was concentrated to ~20 μl under a nitrogen stream and kept at −30°C in a freezer.

Effluvial Collections. Volatiles were collected using four groups of 5- to 20-d-old virgin females (25, 30, 30, and 37 females per group) at room temperature. Moths were introduced separately into three 1-l, 4-necked glass containers (Zhang et al., 1994). Air was drawn into the container through 6–14 mesh activated charcoal (Fisher Scientific, Pittsburgh, PA), and out of the container through two traps (15 cm × 1.5-cm o.d.) containing Super Q (200 mg each; Alltech Associates, Inc., Deerfield, IL) by vacuum (~1 l/min). Female moths were fed with 10% sugar solution on cotton balls and aerated continuously for 3–4 d at room temperature and 16L:8D photoperiod. The adsorbent traps were changed every 24 hr. Adsorbents were eluted with methylene chloride (4 × 0.5 ml); eluates (2 ml/each sample) were concentrated to ~20 μl under a nitrogen stream and stored at −30°C.

Microreductions. A 60-μl hexane solution containing 60 ng of *E*11-16:Ald or 10 female equivalent (FE) of gland extracts in a conical glass vial was treated with 20 μg of $NaBH_4$ (5 μl of 2-propanol solution, 4 mg/ml). The vial was swirled several times, and after 5 min, 5 μl of water was added and the mixture was agitated to hydrolyze the borate esters (Klun et al., 1982). The organic layer was transferred into another conical glass vial and analyzed by GC and GC–EAD.

Electrophysiological Recordings. The coupled gas chromatographic–electroantennographic detection (GC–EAD) system was as previously described (Zhang et al., 1997; Zhang and Polavarapu, 2003). A Hewlett-Packard 6890 gas chromatograph equipped with a 60 m × 0.25-mm i.d., 0.25-μm film-thickness DB-WAXETR capillary column (J&W Scientific Inc., Folsom, CA, 120°C for 2 min, then programmed to 250°C at 10°C/min and held for 10 min) or a 60 m × 0.25-mm i.d., 0.25-μm film-thickness DB-1 capillary column (J&W Scientific Inc., 100°C for 2 min, then programmed to 300°C at 10°C/min and held for

10 min) in the splitless mode with hydrogen as carrier gas (1.4 ml/min) was used for GC–EAD analysis.

Chemicals. (*E*)-11-Hexadecenal was purchased from the Pherobank, Wageningen, The Netherlands (>99% purity). All other synthetic pheromone standards had previously been synthesized and purified in our laboratory (Lynch et al., 1984), and stored in a −30°C freezer. Purities of chemicals were checked on a 60-m polar DB-WAXETR GC capillary column before preparing lures for the field study.

Field Tests. Red natural rubber septa (5 mm, Wheaton, NJ) and gray halobutyl rubber septa (5 mm, The West Company, NE) loaded with the desired rates of *E*11-16:Ald and a mixture of *EZ*-isomers in ~40 μl of hexane solution and 2 drops of butylated hydroxyltoluene (BHT) solution (10 mg/ml hexane) were used for field trials. The same amount of hexane was loaded on each kind of septum for the blank control. After loading, the solvent was allowed to evaporate in a fume hood for 30 min. Lures were wrapped in aluminum foil, stored in 20-ml plastic vials, and shipped by courier. Upon arrival in Chatsworth, NJ, the lures were kept in a freezer at −10°C until used.

All field tests were conducted in commercial blueberry fields near Hammonton, Atlantic County, and Chatsworth, Burlington County, NJ, using Pherocon 1C sticky traps (Trécé, Salinas, CA). Traps were arranged in a randomized complete block design with four or five replicates and 20–25-m intertrap distances within replicates. Replicates were separated by ~22 m (8 rows of blueberries). Traps were hung at crop canopy level ~150 cm above ground level, checked and moved by one position every 3–7 d in different tests, and the bottoms were replaced when catches exceeded 150 moths per trap. Caged live virgin females (2- to 4-d-old, 2 females per cage) were used as positive controls. Females were replaced with fresh insects after each trap check for the duration of the test.

Statistical Analysis. Data on trap catches from each test were square-root-transformed ($\sqrt{\chi} + 0.5$) or logarithm transformed ($\log\chi + 1$) to normalize the variance before analysis. Means were compared by either paired-samples *t*-test (2-tailed) or one-way analysis of variance (ANOVA) followed by Ryan–Einot–Gabriel–Welsch Range test (SPSS 10.0 for Windows) (George and Mallery, 2002) for significance at $\alpha = 0.05$.

RESULTS

Identification of the Antennal Stimulatory Component in Female Gland Extracts and Effluvial Collections. Coupled GC–EAD analyses of female gland extracts and effluvial collections demonstrated that antennae of male *C. porphyretica* consistently responded to a single compound (Figure 1A and B). The EAD-active peaks were observed at 10.97 min on a 60-m DB-WAXETR capillary column and at 12.78 min on a 60-m DB-1 capillary column. The amount of natural

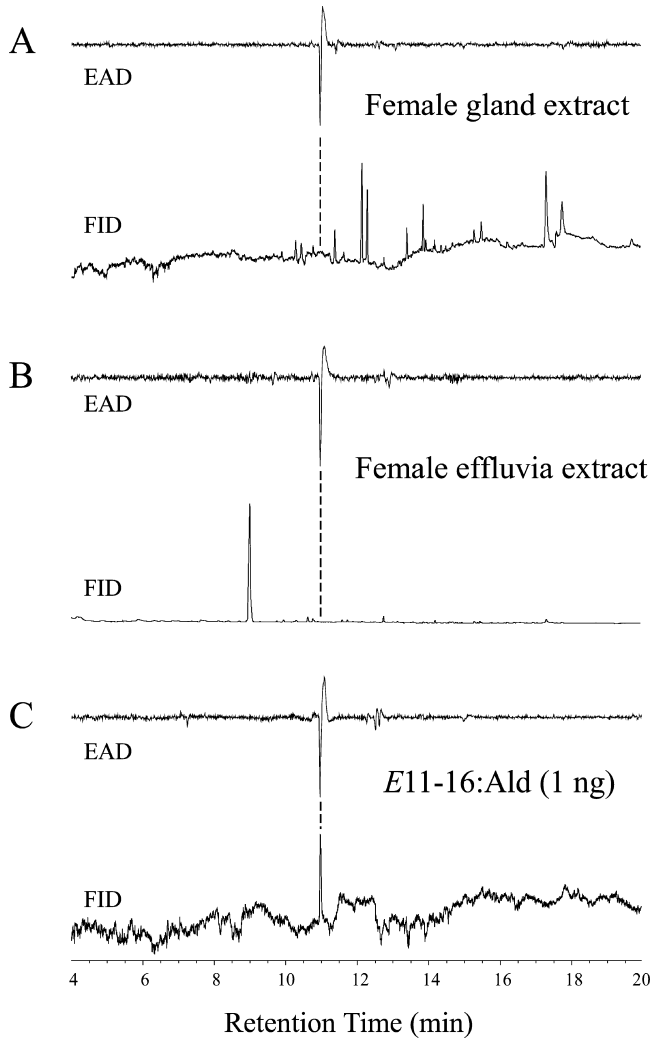

FIG. 1. Simultaneous FID and EAD responses of the antenna of an adult male *C. porphyretica* to (A) a pheromone gland extract (15 FE) from virgin female *C. porphyretica* (5- to 7-d-old); (B) effluvia (25 FE) trapped from virgin female *C. porphyretica* (5- to 20-d-old); and (C) synthetic *E*11-16:Ald (1 ng) on a DB-WAXETR column.

EAD-active component collected from female *C. porphyretica* was below the FID and MS detection thresholds, even with highly concentrated gland extracts (~150 FE). Therefore, identification of the EAD-active component relied on the comparison of the retention times of EAD-active compounds with authentic

standards on polar and nonpolar GC columns, and a microreaction followed by GC–EAD analysis.

In the genus *Caloptilia*, the only other species for which a female sex pheromone has been identified is the tea leafroller, *C. theivora* Walsingham (Ando et al., 1981, 1985). In this species, *E*11-16:Ald was identified as the main pheromone component, but 1–3% of *Z*11-16:Ald was found to be essential for optimum activity. Thus, we tested hexadecanal in GC-EAD analyses, and found that it stimulated strong EAD responses from male antennae. The retention times of 16:Ald at 10.67 min (polar column) and 12.93 min (non polar column) were slightly shorter than the EAD-active peak (10.97 min) on the DB-WAXETR column and slightly longer than the EAD-active peak (12.78 min) on the DB-1 column, suggesting that the pheromone component could be an unsaturated C_{16} aldehyde. The low GC–EAD responses and nonmatching retention times of C_{14} and C_{16} unsaturated acetates ruled out the possibility of acetates being the pheromone candidates (Table 1). When a variety of monounsaturated aldehydes were subjected to GC analyses, EAD-active peaks matched only with the GC retention times of *E*11-16:Ald on both capillary columns (Table 1), and the strong antennal activity of *E*11-16:Ald was confirmed with an authentic standard (Figure 1C).

Antennae of female *C. porphyretica* responded to all the compounds tested (Table 1). When 30 ng of synthetic *E*11-16:OH (>99.9% pure) was subjected to GC–EAD analysis, strong EAD responses were elicited not only from the

TABLE 1. RETENTION TIMES OF EAD-ACTIVE COMPOUND OBTAINED FROM EFFLUVIA AND GLAND EXTRACTS OF FEMALE *C. porphyretica* AND SYNTHETIC STANDARDS

Compounds	Rentention time (min)		EAD activity in 10 ng of loading (mV)
	DB-WAXETR	DB-1	
From females			
(*E*)-11-16:Ald	10.97	12.78	
Synthetic			
16:Ald	10.67	12.93	1.30 (±10.0% SD, $n = 3$)
(*Z*)-7-16:Ald[a]	10.93	12.70	
(*Z*)-9-16:Ald[a]	10.95	12.74	
(*E*)-11-16:Ald	10.97	12.78	2.30 (±10.1% SD, $n = 3$)
(*Z*)-11-16:Ald	11.04	12.81	0.27 (±5.8% SD, $n = 3$)
(*E*)-11-16:OH	13.08	13.48	0.67 (±11.6% SD, $n = 3$)
(*E*)-11-14:Ac	10.62	12.81	0.30 (±10.0% SD, $n = 3$)
(*Z*)-11-14:Ac	10.74	12.87	0.17 (±0.58% SD, $n = 3$)
(*Z*)-12-14:Ac	11.07	13.06	0.30 (±10.0% SD, $n = 3$)
(*E*)-11-16:Ac	12.46	14.71	0.23 (±5.8% SD, $n = 3$)

[a] Compounds not tested by EAD.

*E*11-16:OH (Figure 2A, peak 2), but also from a FID-undetectable residual *E*11-16:Ald contaminant (Figure 2A, peak 1), indicating that male *C. porphyretica* antennae were extremely sensitive to the *E*11-16:Ald.

The identity of *E*11-16:Ald was further verified by treatment of the female gland extracts with $NaBH_4$ to convert the aldehyde to the corresponding alcohol.

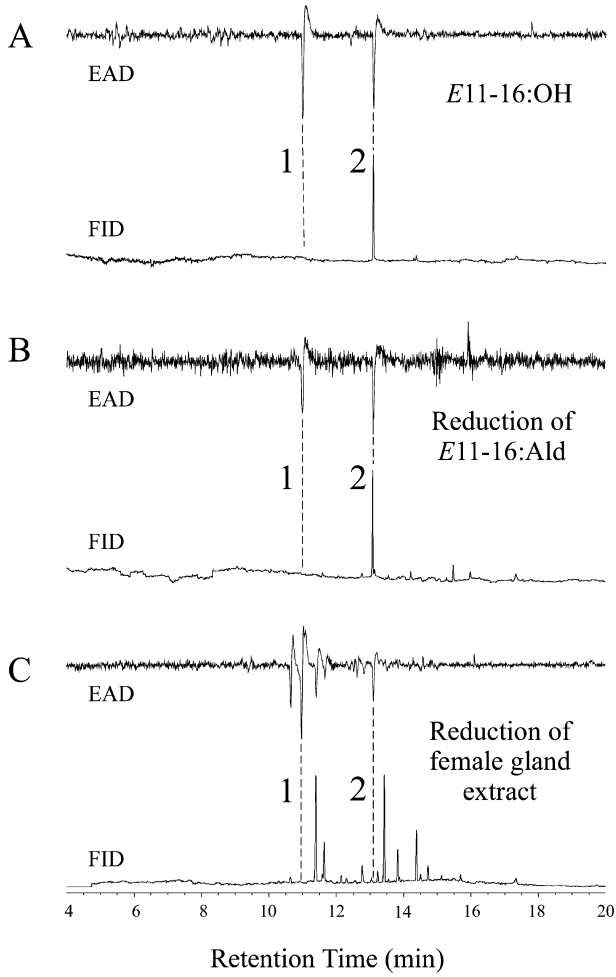

FIG. 2. Simultaneous FID and EAD responses of an adult male *C. porphyretica* antenna to (A) 30 ng of synthetic *E*11-16:OH; (B) $NaBH_4$-reduced *E*11-16:Ald (~10 ng); and (C) $NaBH_4$-reduced gland extracts (5 FE) on a DB-WAXETR column. Identifications of peaks: **1**, *E*11-16:Ald; **2**, *E*11-16:OH.

GC–EAD of the reduced gland extract showed the presence of a new EAD response at the retention time of *E*11-16:OH on both capillary columns (Figure 2C), providing supporting evidence that the corresponding aldehyde, *E*11-16:Ald, was present in the original extract. EAD peak 1 was elicited by traces of unreacted *E*11-16:Ald. Treatment of 30 ng of synthetic *E*11-16:Ald with $NaBH_4$ resulted in similar GC–EAD responses (Figure 2B). Because even trace amounts of residual *E*11-16:Ald in reaction mixtures still evoked large EAD responses, other microreactions were not attempted. In total, the various pieces of information provide strong evidence that *E*11-16:Ald is a female sex pheromone component of *C. porphyretica*.

GC–EAD experiments were conducted with antennae of male *C. porphyretica* challenged with various standards. Among monounsaturated C_{14} and C_{16} standards, aldehydes elicited much stronger EAD responses than other candidate pheromone components (Table 1). In particular, male *C. porphyretica* antennae exhibited ability to discriminate the *Z*- and *E*11-16:Ald isomers. Antennae responded strongly to traces of *E*11-16:Ald when >99% pure *Z*11-16:Ald was subjected to GC–EAD analysis (Figure 3). The GC–EAD experiments also showed that synthetic *E*11-16:Ald elicited unusually strong electrophysiological activity, resulting in significant male antennal responses at doses as low as 10 fg (Figure 4).

Field Trapping Tests. Traps baited with 3 μg of *E*11-16:Ald were remarkably attractive to male *C. porphyretica* in the field. The captures in *E*11-16:Ald-baited traps were influenced by climatic factors during the flight season and other factors, such as insecticide applications. However, trap captures indicated that there were

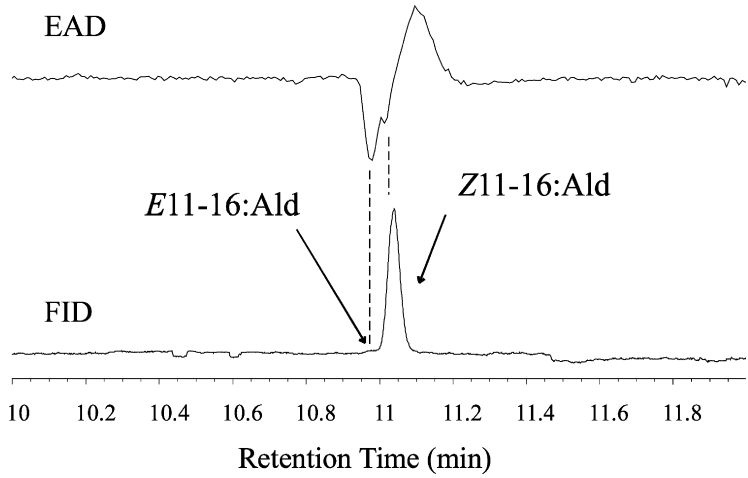

FIG. 3. Simultaneous FID and EAD responses of an adult male *C. porphyretica* antenna to 10 ng of synthetic *Z*11-16:Ald on a DB-WAXETR column.

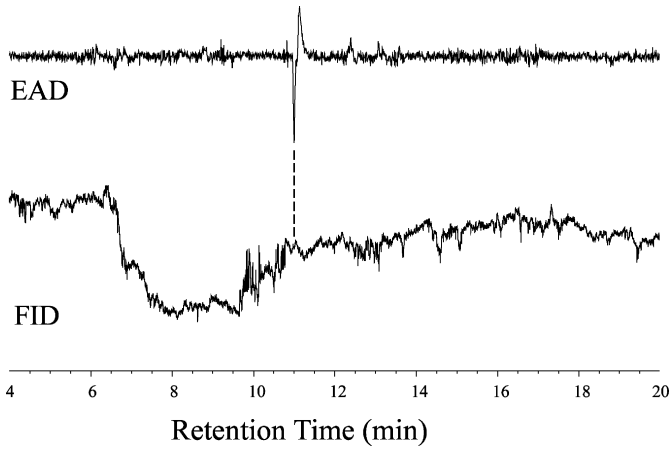

FIG. 4. Simultaneous FID and EAD responses of an adult male *C. porphyretica* antenna to 10 fg of synthetic *E*11-16:Ald on a DB-WAXETR column.

at least three distinct peaks (May 12, July 25, and September 10) separated by periods (~1–2 wk) with few male catches. Considering the time required for egg, larval, and pupal development, there appears to be at least three distinct generations each season (Figure 5).

Initial field tests conducted from July 1 to August 19, 2003, demonstrated that moth catches (total number of male *C. porphyretica* captured was 12,264) were similar in traps baited with gray or red natural rubber septa loaded with 3 μg of *E*11-16:Ald ($t = -1.104$, df = 3, $P = 0.350$). *E*11-16:Ald dose dramatically affected the trap catch of *C. porphyretica*. *E*11-16:Ald doses of 30–100 μg on gray rubber septa resulted in higher trap captures than 0.5–10-μg doses (Figure 6).

To investigate further the efficacy of gray and red rubber septa, we compared these septa loaded with 30–1000 μg of pheromone along with traps baited with two virgin females. Red rubber septa loaded with 1000-μg doses attracted more males than 100-μg and lower doses, whereas catches with 300-μg doses were intermediate (Figure 7). However, with gray rubber septa, moth catches were significantly higher with 1000-μg doses than any other doses tested. Traps baited with gray or red rubber septa loaded with 1000 μg of *E*11-16:Ald attracted similar numbers of males as traps baited with two virgin females (Figure 7). Data from this experiment also demonstrated that, except at the highest dose (1000 μg/septum), red rubber septum lures attracted more males than gray rubber septa loaded with the same doses.

In the final field test, we evaluated the effect of addition of 1–10% of *Z*11-16:Ald to *E*11-16:Ald (Figure 8). Addition of 1 and 3% of the *Z*-isomer to

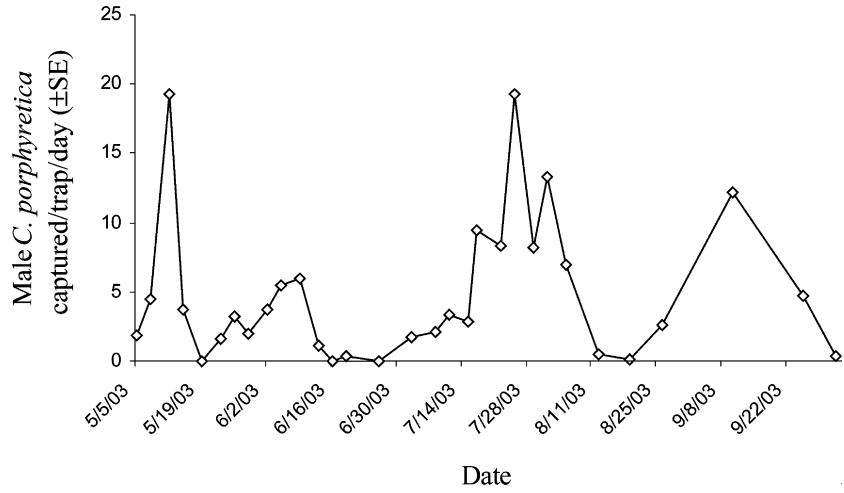

FIG. 5. The flight activity of *C. porphyretica* monitored by Pherocon 1C traps baited with 3 μg *E*11-16:Ald (5/1–7/3 using ~95% pure compound on gray rubber septa and 7/3–10/2 using ~98% pure compound on red rubber septa). The trial was conducted from May 1 to October 2, 2003. Total number of male *C. porphyretica* captured was 2481.

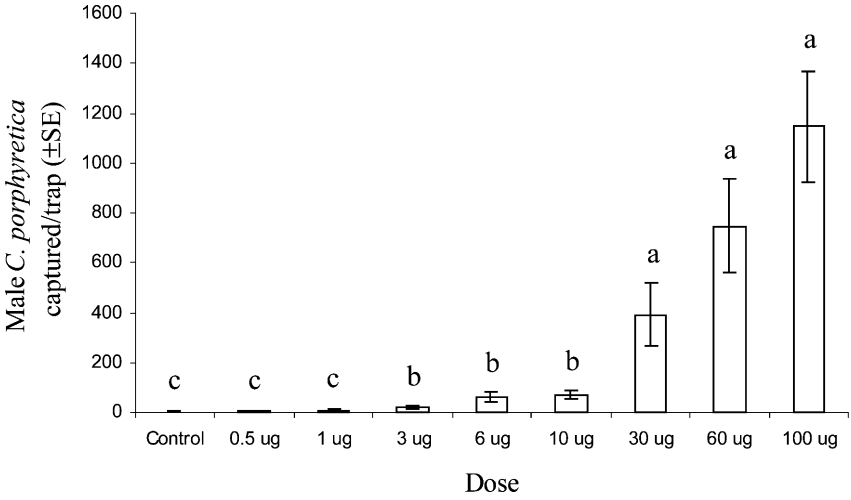

FIG. 6. Results of *C. porphyretica* pheromone dose–response field tests on gray rubber septa. The trial was conducted from July 1 to August 1, 2003. Total number of male *C. porphyretica* captured was 12,264. Bars superscripted by different letters are statistically different (logarithm transformed, $N = 5$, $F_{8,36} = 49.67$, $P < 0.05$).

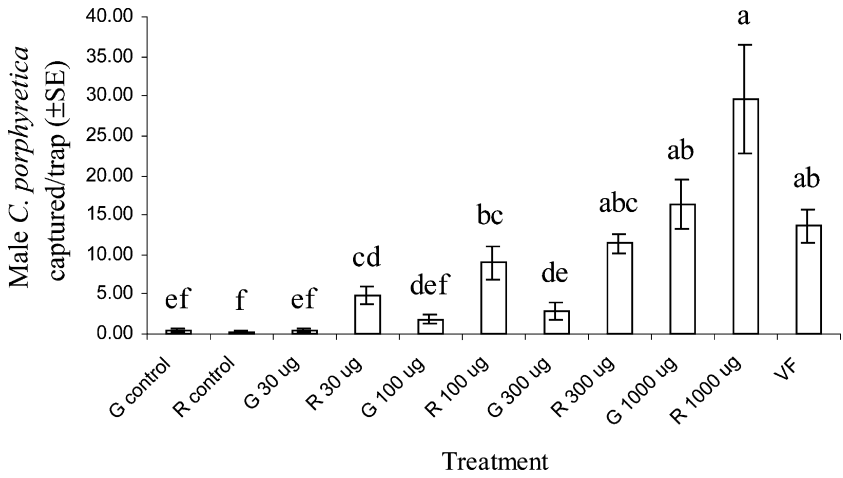

FIG. 7. *C. porphyretica* males captured in traps baited with two virgin females and different amounts of *E*11-16:Ald on two kinds of rubber septa. The trial was conducted from August 29 to September 23, 2003. Total number of male *C. porphyretica* captured was 452. Bars superscripted by different letters are statistically different (logarithm transformed, $N = 5$, $F_{10,44} = 28.07$, $P < 0.05$). G = gray halo-butyl rubber septa, R = red natural rubber septa, and VF = two virgin females.

*E*11-16:Ald (100-μg dose) did not increase trap catches compared with traps baited with 100 μg of *E*11-16:Ald alone. However, as the proportion of *Z*-isomer increased to 10%, male captures decreased compared with captures in traps baited with 0, 1, and 3% of the *Z*-isomer.

DISCUSSION

On the basis of GC-EAD analyses of gland extracts, effluvial collections, microreaction of gland extracts, and field trapping studies, we conclude that *E*11-16:Ald is the EAD-active compound produced by female *C. porphyretica*. Although *Z*11-16:Ald was also electrophysiologically active, addition of 1–3% of this compound to *E*11-16:Ald had no effect on trap captures, suggesting that this compound may not be part of the sex communication system of *C. porphyretica*.

Virgin females under laboratory conditions called during the first 2 hr of the scotophase. The EAD responses of gland extracts collected during photophase were identical to effluvial collections made over a 3-d period, suggesting that glands during photophase contained the same active compound. One possible reason that the amounts of pheromone recovered from gland extracts were so low is that the moths were dissected outside their normal calling period.

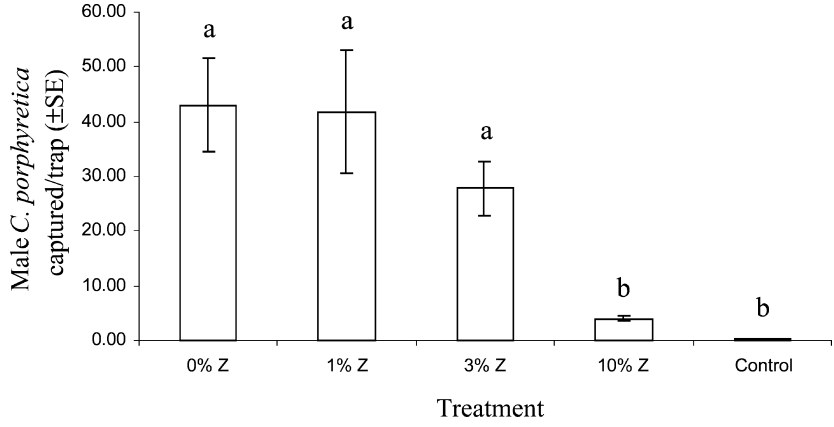

FIG. 8. Effect of addition of Z11-16:Ald to E11-16:Ald on captures of *C. porphyretica* male moths. Amounts of pheromone were 100 μg/septum. Percentage of Z11-16:Ald on the x-axis represents additional amounts of Z11-16:Ald added to 100 μg of E11-16:Ald. The trial was conducted from August 29 to September 23, 2003. Total number of male *C. porphyretica* captured was 584. Bars superscripted by different letters are statistically different (square-root-transformed, $N = 5$, $F_{4,20} = 21.71$, $P < 0.05$).

E11-16:Ald was first identified as a sex attractant for male moths in several lepidopteran families by systematic field screening tests (Ando et al., 1981). This compound has also been reported as a pheromone component of crambid moths, *Diaphania nitidalis* (Klun et al., 1986), and *D. hyalinata* (L.) (Raina et al., 1986), the sphingid moth *Deilephila elpenor* (Bestmann et al., 1992), and the pyralid moths, *Cryptoblabes gnidiella* (Anshelevich et al., 1993) and *Neoleucinodes elegantalis* (Cabrera et al., 2001).

In Lepidoptera, the use of a species-specific blend of pheromone components for sexual communication is more pervasive than the use of single compounds (Arn, 2000). However, in the family Gracillariidae, single-component pheromones appear to be more common. For example, (8E,10E)-tetradecadienal has been reported as a sex pheromone component in *Acrocercops* spp. (Ando et al., 1987). Its geometric isomer, (8E,10Z)-tetradecadienal, is a sex pheromone component of horse chestnut leafminer, *Cameraria ohridella* Deschka & Dimic (Svatos et al., 1999; Francke et al., 2002; Kalinová et al., 2003). *Gracillaria elongella* Linnaeus releases (10Z, 12E)-hexadecadienal (Arn, 2000) and *G. syringella* Fabricius emits (E)-11-tetradecenal that attracts males (Booij and Voerman, 1985). Furthermore, (4E,10E)-dodecadienyl acetate has been discovered in female pheromone gland extracts of tentiform leafminer, *Phyllonorycter mespilella* (Huebner) (Gries et al., 1993). Female *P. platani* emit (Z)-10-tetradecenyl acetate for mate location (Subchev et al., 2003), and the same compound has been found from abdominal

tip extracts of the tentiform leafminer moth *P. ulmifoliella* (Mozuraitis et al., 1997). Finally, (*E*)-10-dodecenyl acetate has been used for monitoring as well as mating disruption of spotted tentiform leafminer, *P. blancardella* (Fabr.) (Trimble and Tyndall, 2000).

Gray halobutyl rubber septa were reported to be better for stabilizing unsaturated pheromone components than red natural rubber septa (Brown and McDonough, 1986). However, on the basis of two different field experiments, we did not see significant differences in moth catches in traps baited with gray and red rubber septa loaded at very low (3-μg) or very high (1000-μg) rates (Figure 7), but red rubber septa attracted significantly more moths at 30–300-μg doses compared with gray rubber septa at the same doses (Figure 7).

Within limits trap catches usually increase with increasing pheromone loading (Carde and Elkinton, 1984; Polavarapu and Seabrook, 1992; Facundo et al., 1994). When doses were increased from 0.5 to 100 μg on gray septa, mean trap capture increased over three orders of magnitude and no inhibitory effects were observed (Figure 6). The larger trap catches observed with increasing doses of *E*11-16:Ald in different experiments (Figures 6 and 7) might be attributed to expanded active space of the trap and overlap of active spaces, causing male moths to preferentially seek traps baited with higher doses upwind (Wall and Perry, 1987). Furthermore, moth captures were comparable in traps baited with two virgin females and traps baited with red rubber septa baited with 100–1000-μg doses, indicating the suitability of doses in this range for population monitoring.

Overall, the electrophysiological, chemical, and behavioral data reported here all support the identity of *E*-11-16:Ald as the main pheromone component of *C. porphyretica*. (*E*)-11-Hexadecenal at doses of 100–300 μg on red natural rubber septa should provide an effective lure for monitoring populations of this insect.

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BEHAVIORAL RESPONSE OF *Lygus hesperus* TO CONSPECIFICS AND HEADSPACE VOLATILES OF ALFALFA IN A Y-TUBE OLFACTOMETER

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Abstract—The western tarnished plant bug, *Lygus hesperus* Knight, feeds and develops on a variety of weeds in the spring, with later generations moving to alfalfa and cotton where severe damage to reproductive structures can occur. A synthetic attractant for monitoring or mass-trapping *L. hesperus*, or the identification of potential attractants for natural enemies, would be useful tools for integrated pest management programs. Studies investigated the response of naive and experienced fifth-instar and adult *L. hesperus* to odors associated with conspecifics and alfalfa, *Medicago sativa* L. Fifth-instar *L. hesperus* responded to all plant/insect combinations, whereas female *L. hesperus* only responded preferentially to vegetative and flowering alfalfa where conspecifics had fed for 24–72 hr, and to vegetative alfalfa where conspecifics were added approximately 30 min before the test began. Males were not attracted to headspace volatiles from any of the alfalfa treatments. Analysis of headspace volatiles showed that (*E*)-2-hexanal, (*Z*)-3-hexen-1-ol, α-pinene, (*Z*)-3-hexenyl acetate, (*E*)-2-hexenyl acetate, limonene, (*Z*)-ocimene, (*E*)-β-ocimene, linalool, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, and (*E*, *E*)-α-farnesene are emitted from both vegetative and flowering alfalfa. Indole and (3*E*, 7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene were only detected in flowering alfalfa. Damage to alfalfa by *L. hesperus* increased emissions of (*Z*)-ocimene, (*E*)-β-ocimene, (*E*)-β-caryophyllene, and (*E*, *E*)-α-farnesene, while β-pinene, myrcene, methyl salicylate, and (3*E*, 7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene were only detected from damaged plants. Thus, individual or mixtures of these alfalfa volatiles may be useful

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as attractants for capturing nymphs and adult females of *L. hesperus* in the field.

Key Words—Miridae, western tarnished plant bug, Y-tube olfactometer, alfalfa, host location, herbivore-induced volatiles

INTRODUCTION

Lygus hesperus Knight (Heteroptera: Miridae), the western tarnished plant bug, is the most common species of *Lygus* bug found in the western United States. It is an extremely polyphagous species found on over 100 species of plants in 24 families (Scott, 1977). Many important crops such as beans, strawberries, peaches, cotton, and various seed crops are damaged by this insect. *Lygus* bugs prefer to feed on the meristematic and developing reproductive tissues of their hosts, which causes abortion of buds, blooms, and fruits, destruction of seeds or ovules, deformation of fruits, initiation of abnormal secondary vegetative growth, and necrotic spotting of fruits (Strong, 1970; Leigh, 1976; Mauney and Henneberry, 1984; Leigh et al., 1988). Although *L. hesperus* is also a facultative predator of insects (Wheeler, 1976; Agusti and Cohen, 2000), potential benefits are outweighed when large numbers move into crops where the preferred feeding habit might be herbivory.

A synthetic attractant would be a useful tool for monitoring or mass trapping *Lygus* spp. Most efforts to develop an attractant have focused on identifying sex pheromones of plant bugs (Gueldner and Parrott, 1978; Aldrich et al., 1988; Chinta et al., 1994; Groot et al., 1999; Ho and Millar, 2002), and although likely components have been determined, responses to these compounds in the field have been disappointing (Hedin et al., 1985; McLaughlin, 1998; Ho and Millar, 2002). A complimentary approach would be to identify the host-plant cues that attract *Lygus* spp., which in turn could be used in combination with visual sticky traps. Additionally, as herbivore-induced volatiles enhance the foraging success of many natural enemies (Dicke and Sabelis, 1988; Turlings et al., 1990; Agelopoulus and Keller, 1994; Powell et al., 1998), a better understanding of *Lygus*-plant interactions could lead to novel ways of using attractants to control this pest. Alfalfa, *Medicago sativa*, was chosen for our study because it is a preferred host for *L. hesperus*, and most likely to provide compounds that would be highly attractive to this species (Sevacherian and Stern, 1974, 1975; Whitbey, 1999).

The present study was undertaken to determine whether headspace volatiles released by alfalfa are used by fifth-instar and adult *L. hesperus* during host location. We also were interested in establishing whether volatiles associated with conspecifics feeding on alfalfa enhanced or inhibited the host-location response, and whether experienced *L. hesperus* were better at locating the host plant than naive individuals. Furthermore, volatile constituents relative to plant phenology

(vegetative or flowering), time of day, and insect damage were identified and their potential roles discussed.

METHODS AND MATERIALS

Insect Rearing and Maintenance. Lygus hesperus nymphs and adults were collected from alfalfa fields located at The University of Arizona—Maricopa Agricultural Center, Maricopa, AZ. To maintain genetic diversity, feral individuals were added to the colony 3–4 times per year. Green beans, carrots, pink bollworm eggs, and 10% sucrose solution were provided as food, and were changed every other day. The green beans and carrots also served as oviposition substrates, which were placed in 2 × 14-cm diam Petri dishes lined with filter paper, and then maintained in an incubator until first-instar *L. hesperus* emerged. Newly emerged nymphs were placed in 8.5 × 12.5-cm diam paper cartons where the center of each lid had been replaced with nylon organdy to allow air circulation. Nymphs were maintained with food and sucrose solution until they were needed for experiments. Insects were maintained in an incubator at 23 ± 2°C, 55 ± 15% RH, under a light–dark regime of 14L:10D.

Plant Maintenance. Medicago sativa (cv.'Cuf 101') was planted in 1-l pots containing a standard soil mixture and maintained in a greenhouse at 25 ± 5°C and 50–85% RH. Natural lighting provided a light-dark regime of 12L:12D during the trials. Plants were watered and fertilized regularly by means of a drip irrigation system. A 1:1 mixture of all-purpose Scotts Miracle-Gro Excel (21-5-20) and cal-mag Miracle-Gro Professional (15-5-15) was applied at a rate of 1/100 l of water.

Y-tube Olfactometer Setup. Bioassays were conducted in a 40-mm diam × 36-cm long glass, Y-tube olfactometer that had 50° inside angle. Incoming air was filtered through activated charcoal and humidified with doubly distilled, deionized water. The filtered air was split between two, 2-l holding chambers; one chamber served as a control (clean air) and the other chamber held the test material (i.e., plant or plant + insect). From each holding chamber, the air passed into the respective arms of the Y tube, and then through a series of screens before entering the main tube of the olfactometer. Airflow through the system was maintained at 4.8 l/min (=3.8 m/min inside the tube) by an inline flowmeter (Gilmont Instr., Barnant Co., Barrington, IL.). A smoke test demonstrated laminar airflow in both arms and throughout the olfactometer.

A 60-cm long, wide-spectrum fluorescent lamp (GE, F20T12-PL/AQ) was positioned 22 cm above the arms of the Y tube. Before each trial, light intensity over each arm was measured with a light meter (ExTech Instr. Model 401025, Zefon International, St. Petersburg, FL), and the tube was adjusted until intensity was the same in both arms. Light intensity averaged 810.6 ± 7.2 (mean ± SE) lux. The Y-tube setup was surrounded by a 50 × 70 × 60-cm black fabric enclosure, and

the holding chambers containing the treatments were placed outside the enclosure to eliminate visual cues. Holding chambers were illuminated by 40-W incandescent bulbs, which provided approximately 3000 lux to the plant material during the trials.

Bioassays. Approximately 30 min before trials were initiated, fifth-instar or 7- to 10-d-old adult *L. hesperus* were placed into individual holding/release tubes. Each tube was constructed from a 15-cm long, 5-ml plastic pipette from which 0.5 cm of the bulb and 8 cm of the pipette tip were removed. The cut end of the pipette tip was covered with organdy. A nymph or adult was placed inside the tube, and the end where the bulb tip had been removed was sealed with a cork. Tubes containing bugs were then placed into a separate holding container, so they would not be exposed to test odors before their release. Experienced nymphs and adults were obtained by allowing insects to feed on plants that were the same age as the treatment for 24–72 hr before being placed into the holding/release tubes.

Plants, with or without *L. hesperus*, were then placed into one of the holding chambers. For vegetative alfalfa treatments, we used 30-cm tall, intact plants in which their root system was wrapped with moist paper towels and then placed inside a plastic sleeve. Because plants are much larger (0.5 m^3) by the time they begin to flower, we used five stems, which were cut 30 cm below the flowers and treated as indicated above for vegetative plants. Headspace volatile profiles of these vegetative and flowering alfalfa treatments were comparable (in terms of compounds detected and relative amounts) to the headspace volatiles from greenhouse collections (unpublished data). For the treatments that consisted of plants plus conspecifics, we used a 1:1 sex ratio of adults, when adults were tested, and similar aged nymphs, when nymphs were tested. For longer term (24–72 hr) feeding treatments, we placed adults or nymphs inside fine-mesh bags, on the plants that were to be tested. Halfway through each olfactometer trial, fresh plant material was placed in the holding chamber, and the chamber was moved to the opposite side of the Y-tube setup. This eliminated any potential bias due to odor-source location.

At the beginning of each trial, the cork was removed from the holding/release tube, and the open end was placed at the downwind end of the Y tube. Each insect was given 5 min to respond to the treatment, and a choice for the left or right arm of the olfactometer was noted when the insect went 1 cm past the Y junction. The following measurements were recorded for all individuals: time when insect exited the release tube, percentage leaving the release tube, percentage that walked upwind and selected an arm of the Y tube, and response time to first choice. Temperature and RH in the olfactometer were maintained at 24.9 $\pm$ 0.1°C and 80.5 $\pm$ 8.5%, respectively.

Trials usually consisted of 20 insects that were tested only once, and for each individual a clean Y tube was used. Trials were replicated until plant or plant/insect treatments had a minimum of 60 individuals that had responded. The

null hypothesis that *L. hesperus* showed no preference for either olfactometer arm (a response equal to 50:50) was analyzed with a Chi-square goodness of fit test after correcting for continuity with Yates' correction factor (Zar, 1984). Time required for nymphs and adults to exit the release tube, percentage leaving the release tube, percentage that walked upwind to an arm, and response time to first choice for controls versus treatments were compared by two-way ANOVAs. Count data were transformed using square-root or log transformations and percentage data were arcsine transformed to meet the requirements of normality and homogeneity of variance before analysis.

Headspace Volatile Collections. To determine the types of compounds that *L. hesperus* might use to locate a host plant, we investigated the effects of plant phenology (vegetative or flowering), time of day, and insect damage by *L. hesperus* nymphs and adults on volatile emissions in alfalfa. Volatiles from vegetative and flowering alfalfa were collected using a push/pull apparatus constructed after Heath and Manukian (1994), and described in detail in Rodriguez-Saona et al. (2001), which allowed for simultaneous collections from four different plants. Plants were maintained in an air-conditioned greenhouse under natural light (~55,000 lux) and Arizona summer–autumn conditions (12L:12D regime, and 28°C day, 24°C night). On each sampling date, five to 10 stems from each plant were inserted into each glass cylinder (42.5 cm high and 18 cm diam; Analytical Research Systems Inc., Gainesville, FL), cotton was packed around the stems, and the cylinders were sealed off with guillotine-like bases that contained circular openings for the stems. Moist and dry air passed over the plants within the cylinders at a rate of 3 l/min. Near the base of the glass cylinders, eight openings allowed for the attachment of Super Q adsorbent collection traps (Alltech Assoc. Inc., Deerfield, IL). The tip of each collection trap was placed a few millimeters from the plant, and air was pulled through the trap at a rate of 1 l/min.

To test for diurnal variation in volatile emissions from vegetative and flowering alfalfa, Super Q traps were collected and processed at 4-hr intervals during the day (06:00–10:00, 10:00–14:00, and 14:00–18:00 hr). A total of eight vegetative and six flowering plants were sampled. In a second experiment, we tested the effect of damage by *L. hesperus* on volatile emissions from vegetative and flowering alfalfa. Thirty 4–5th instar nymphs or adults (7–10 days old; 1:1 sex ratio) were allowed to feed on the plant material for 48–72 hr before collecting headspace volatiles. These time periods and densities of *L. hesperus* are sufficient to induce volatile emissions in plants (Rodriguez-Saona et al., 2002). Nymphs and adults remained on the plant material during aeration. Volatiles were collected for eight consecutive hours (09:00–17:00 hr). Volatiles from damaged and undamaged plants were collected concurrently on a particular date. Each treatment was replicated 4–7 times.

After volatile collections, traps were rinsed with 180 μl of methylene chloride for extraction of the volatile compounds. Samples (1 μl) were analyzed with a

Hewlett-Packard gas chromatograph (GC model 6890) equipped with an HP1 methyl siloxane column (30 m × 0.32 mm ID, 0.25 μm film), a capillary injector system, and a flame ionization detector. Helium was used as the carrier gas at a linear flow velocity of 40 cm/sec. After injection, column temperature was maintained at 50°C for 3 min, increased by 5°C/min to 190°C, and then maintained at this temperature for 5 min. Individual compounds in the blend were identified using synthetic standards from commercial sources and by GC-mass spectroscopy (as described by Rodriguez-Saona et al., 2001). Spectral data were compared with spectra from the National Institute of Standards and Technology (NIST, 1995) database.

Amounts of each compound (ng/hr) were estimated by comparison of their peak areas with that of the internal standard (600 ng of *n*-octane in 5 μl of methylene chloride). Although this method to analyze plant volatiles does not take into account the fact that different compounds with the same amount might have different responses in terms of GC areas, it has commonly been used to compare volatile emissions between insect-damaged and undamaged plants (e.g., Turlings et al., 1998; Rodriguez-Saona et al., 2002). The effects of plant phenology (vegetative and flowering) and time of day (06:00–10:00, 10:00–14:00, and 14:00–18:00 hr) on volatile emissions in alfalfa were analyzed by using two-way MANOVA (SYSTAT, 1998). Similarly, the effect of damage by *L. hesperus* nymphs and adults on volatile emissions from vegetative and flowering alfalfa was analyzed with MANOVA. Amounts were log transformed before analyses to meet the assumptions of normality and homogeneity of variance. Compounds were classified based on their biosynthetic origin, so that lipoxygenase products (which include the so-called green-leaf volatiles = GLVs), monoterpenes, homoterpenes, and sesquiterpenes were analyzed as groups (*sensu* Paré and Tumlinson, 1998). In the same manner, indole and methyl salicylate (shikimic acid pathway) were analyzed as a group. Only groups containing a minimum of two compounds sharing the same biosynthetic pathway were considered for MANOVA. The insect-produced hexyl butyrate (Ho and Millar, 2002), was not included in the analysis.

RESULTS

Bioassays. The response of naive and experienced fifth-instar *L. hesperus* was similar ($t = -0.002$, df = 14, $P = 0.99$), so data were pooled before Chi-square analyses were performed. For fifth-instar *L. hesperus*, volatile compounds emitted from plant and plant-insect combinations were always preferred over clean air ($P < 0.05$ in all cases; Figure 1). The strongest upwind responses were to vegetative alfalfa on which nymphs had fed for 24–72 hr, and to flowering alfalfa where nymphs were added approximately 30 min before the test began (86.6 and 81.1 % responded to treatments, respectively).

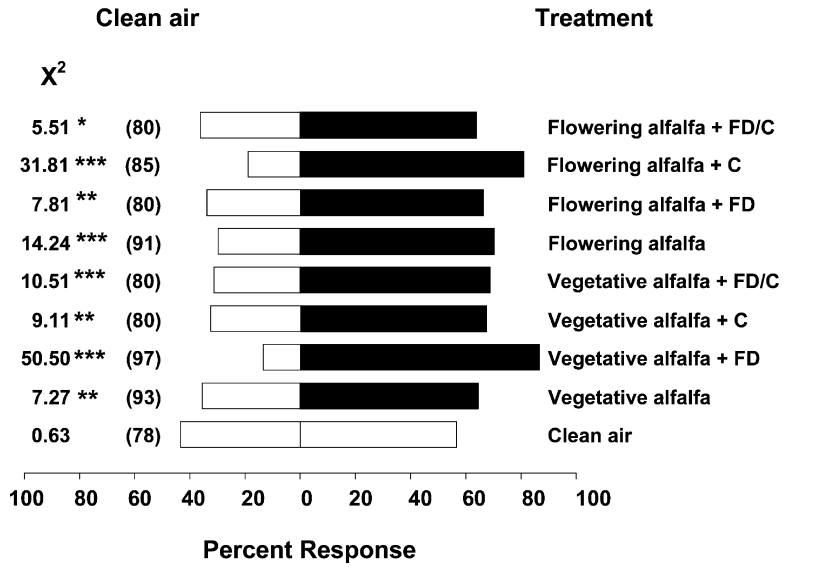

FIG. 1. Response of fifth-instar *Lygus hesperus* to headspace volatiles associated with vegetative and flowering alfalfa with or without feeding damage (FD) and conspecifics (C). Number in parentheses represents sample size; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Similar to nymphs, previous exposure to the treatments did not enhance or inhibit adult response ($t = -0.27$, df $= 14$, $P = 0.79$ for males; $t = -0.16$, df $= 14$, $P = 0.87$ for females) so data for naive and experienced adults were pooled before analyses. Females exhibited a significant response to vegetative and flowering alfalfa on which conspecifics had fed for 24–72 hr, and to vegetative alfalfa where conspecifics were added before the test began (63.5, 71.0, and 65.3 % responded to treatments, respectively; Figure 2). Males, however, never exhibited a significant preference to any of the plant or plant-insect combinations when compared to clean air (Figure 3). Male response was significantly inhibited by the vegetative and flowering alfalfa treatments, and to a lesser extent, by vegetative alfalfa on which conspecifics had fed for 24–72 hr.

In terms of their behavioral response to the various treatments, exit time from the release tube was significantly influenced by the insect stage and sex. Males exited the release tube after approximately 13 sec, females after 23 sec, and nymphs after 45 sec (Table 1A). The percentage of bugs that left the release tube was similar (94%) regardless of the treatment; however, the stage of the insect was important (Table 1B). Only 85 % of the nymphs left the release tubes, while 97% of the adults exited. The propensity to walk upwind and make a choice was influenced by the stage of the insect. Only 44% of the nymphs made a choice,

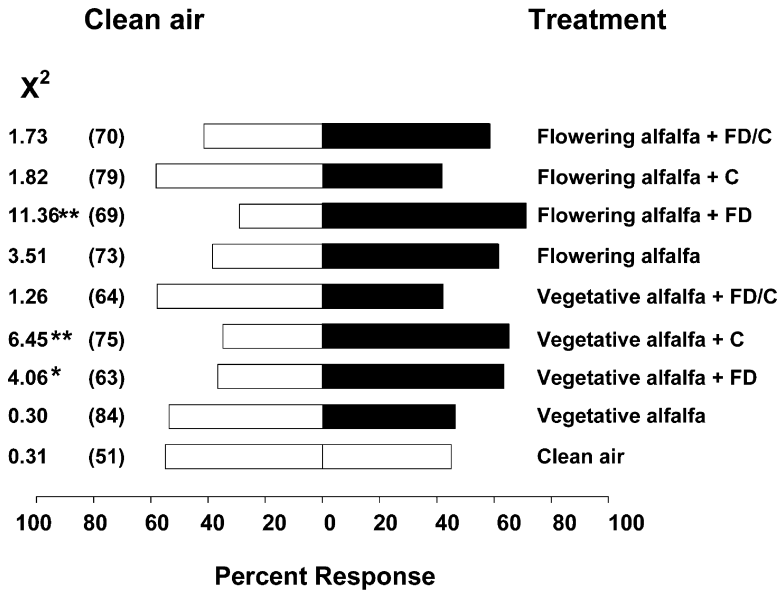

FIG. 2. Response of female *Lygus hesperus* to headspace volatiles associated with vegetative and flowering alfalfa with or without feeding damage (FD) and conspecifics (C). Number in parentheses represents sample size; $^{*}P < 0.05$; $^{**}P < 0.01$.

while 80 % of the females and 82% of the males made a choice between arms of the olfactometer (Table 1C). The time required to make a choice was not influenced significantly by the treatment, but stage was critical (Table 1D). Nymphs made their first choice in 169 sec, females required approximately 108 sec, and males chose after 75 sec.

Headspace Volatile Collections. All three factors that we evaluated: plant phenology (vegetative or flowering), time of day, and insect damage by *L. hesperus*, affected volatile emissions in alfalfa (Tables 2 and 3; Figures 4 and 5). Flowering alfalfa emitted higher amounts of volatiles as compared to vegetative alfalfa (Tables 2 and 3, MANOVA, significant Plant effect, Figures 4 and 5). Compounds such as (*Z*)-3-hexenyl butyrate from the lipoxygenase pathway, the monoterpenes α-pinene, limonene, (*Z*)-ocimene, (*E*)-β-ocimene, and linalool were emitted in higher quantities from flowering alfalfa. Indole and the homoterpene (3*E*, 7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene were only detected from flowering alfalfa (Figures 4 and 5).

Volatile emissions in alfalfa were also affected by time of day (Table 2; significant Time effect, Figure 4). Most compounds were emitted in larger quantities from 06:00–14:00 hr, and tended to decline from 14:00–18:00 hr (Figure 4).

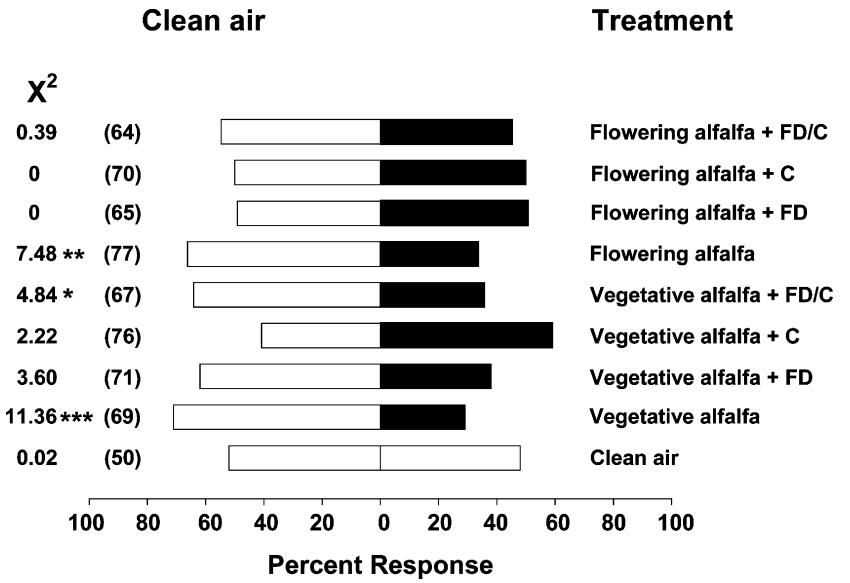

FIG. 3. Response of male *Lygus hesperus* to headspace volatiles associated with vegetative and flowering alfalfa with or without feeding damage (FD) and conspecifics (C). Number in parentheses represents sample size; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

However, there was a clear pattern of emissions for each volatile group (Table 2). Lipoxygenase products such as (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate were emitted in larger amounts soon after the beginning of photophase (06:00–10:00 hr), while several terpenes were emitted in larger amounts at midphotophase, from 10:00–14:00 hr. Compounds specific to flowering alfalfa were also emitted in larger quantities from 10:00–14:00 hr (Figure 4). This latter time interval was used to conduct our behavioral bioassays.

Damage to alfalfa by *L. hesperus* feeding increased volatile emissions (Table 3, significant Damage effect, Figure 5). Damage by adults caused greater induced responses in alfalfa as compared to nymphs. *Lygus* feeding caused increased emissions of the monoterpenes (*Z*)-ocimene, (*E*)-β-ocimene, and linalool, and the sesquiterpenes (*E*)-β-caryophyllene and (*E*,*E*)-α-farnesene. However, the effects of damage on sesquiterpene emissions were dependent on plant phenology (significant Plant $\times$ Damage interaction; Table 3). Damage by *L. hesperus* increased sesquiterpene emissions in vegetative but not flowering alfalfa. Compounds detected only from damaged plants included β-pinene, myrcene, methyl salicylate, and (3*E*, 7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. The *L. hesperus*-produced volatile, hexyl butyrate, also was detected in large quantities when adults were present (data not shown).

TABLE 1. TWO-WAY ANOVAS FOR EXIT TIME(S), PERCENTAGE EXITING RELEASE TUBE, PERCENTAGES THAT PROGRESSED UPWIND, AND TIME TO FIRST CHOICE(S) FOR FIFTH-INSTAR, AND MALE AND FEMALE *L. hesperus*

Source of variation	df	Mean square	*F* ratio	*P*
A. Treatment[a]	1	1.0	0.9	0.4
Stage[b]	2	56.8	51.64	<**0.001**
Interaction	2	0.3	0.3	0.8
Residual	66	2.8		
Total	71			
B. Treatment[a]	1	0.00005	0.003	1.0
Stage[b]	2	0.5	33.1	<**0.001**
Interaction	2	0.02	1.2	0.3
Residual	66	0.03		
Total	71			
C. Treatment[a]	1	0.002	0.1	0.8
Stage[b]	2	1.2	40.2	<**0.001**
Interaction	2	0.004	0.2	0.9
Residual	66	0.1		
Total	71			
D. Treatment[a]	1	22.8	0.05	0.8
Stage[b]	2	51294.7	107.8	<**0.001**
Interaction	2	396.5	0.8	0.4
Residual	66	475.5		
Total	71			

Note. Bold type indicates significant treatment effects ($P < 0.05$).
[a] Treatment refers to clean air versus plant and plant–insect combinations.
[b] Stage refers to fifth-instar or adult (male and female) *L. hesperus*.

DISCUSSION

Our data demonstrate that developmental stage and sex of *L. hesperus* influence their responsiveness and attractiveness to alfalfa odors. In our trials, nymphs that progressed upwind were more responsive to odors emanating from plant and plant-insect combinations than adults (Figures 1–3). The fact that nymphs are capable of making such choices would enable them to locate their host if they became dislodged, which would be critical as their dispersal ability is limited. For the relatively large percentage of nymphs that did not walk upwind, we suspect that the physiological state of the nymph greatly influenced its response. In several instances, nymphs molted shortly after the bioassay ended, and these individuals never moved upwind. Additionally, the type of food consumed (i.e., pink bollworm eggs versus plant material), time since last meal, and size of the meal could influence their tendency to respond to plant odors. The nymph's slower upwind progress, which would make them more susceptible to predation under

TABLE 2. TWO-WAY MANOVA FOR EFFECTS OF PLANT PHENOLOGY (VEGETATIVE AND FLOWERING) AND TIME OF DAY (MORNING: 06:00–10:00 HR; MIDDAY: 10:00–14:00 HR; AND AFTERNOON: 14:00–16:00 HR) ON ALFALFA VOLATILE EMISSIONS

	Plant			Time			Plant × Time		
	Wilks' λ	*F*	*P*	Wilks' λ	*F*	*P*	Wilks' λ	*F*	*P*
Lipoxygenase products[a]	0.674	3.381	**0.014**	0.383	4.309	**<0.001**	0.936	0.235	0.992
Monoterpenes[b]	0.416	9.825	**<0.001**	0.596	2.068	**0.039**	0.627	1.842	0.069
Sesquiterpenes[c]	0.965	0.690	0.508	0.797	2.285	0.068	0.922	0.782	0.540

Note. Bold type indicates significant treatment effects ($P < 0.05$).

[a] Lipoxygenase products include (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexenyl acetate, (*E*)-2-hexenyl acetate, and (*Z*)-3-hexenyl butyrate.

[b] Monoterpenes include α-pinene, limonene, (*Z*)-ocimene, (*E*)-β-ocimene, and linalool.

[c] Sesquiterpenes include (*E*)-β-caryophyllene and (*E*, *E*)-α-farnesene.

TABLE 3. TWO-WAY MANOVA FOR EFFECTS OF PLANT PHENOLOGY (VEGETATIVE AND FLOWERING) AND FEEDING DAMAGE BY *Lygus hesperus* (NYMPHS AND ADULTS) ON ALFALFA VOLATILE EMISSIONS

	Plant			Damage			Plant x Damage		
	Wilks' λ	*F*	*P*	Wilks' λ	*F*	*P*	Wilks' λ	*F*	*P*
Lipoxygenase products[a]	0.532	4.049	**0.009**	0.673	1.007	0.452	0.528	1.728	0.103
Monoterpenes[b]	0.184	13.277	**<0.001**	0.282	2.653	**0.007**	0.466	1.397	0.197
Homoterpenes[c]	0.848	2.323	0.118	0.783	1.690	0.166	0.690	2.652	**0.043**
Sesquiterpenes[d]	0.788	3.499	**0.045**	0.664	2.954	**0.028**	0.684	2.716	**0.040**
Shikimic acid pathway[e]	0.212	48.286	**<0.001**	0.637	3.290	**0.018**	0.729	2.223	0.079

Note. Bold type indicates significant treatment effects ($P < 0.05$).

[a] Lipoxygenase products include (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexenyl acetate, (*E*)-2-hexenyl acetate, and (*Z*)-3-hexenyl butyrate.

[b] Monoterpenes include α-pinene, β-pinene, myrcene, limonene, (*Z*)-ocimene, (*E*)-β-ocimene, and linalool.

[c] Homoterpenes include (3*E*)-4,8-dimethyl-1,3,7-nonatriene and (3*E*, 7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene.

[d] Sesquiterpenes include (*E*)-β-caryophyllene and (*E*, *E*)-α-farnesene.

[e] Shikimic acid pathway include indole and methyl salicylate.

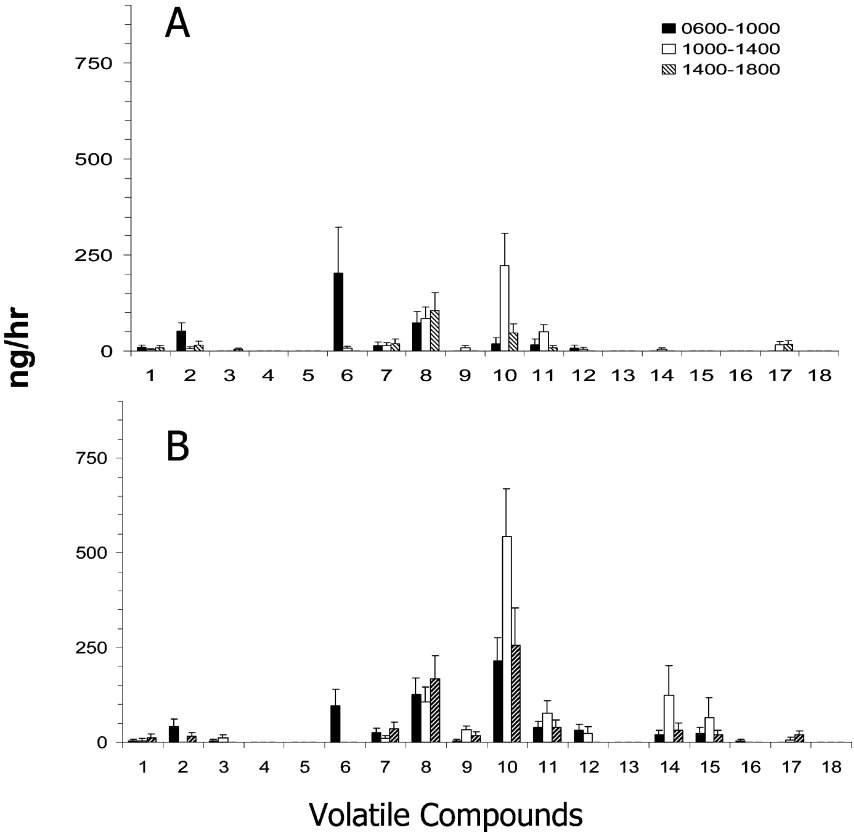

FIG. 4. Volatiles collected from the aerial portions of vegetative (A) or flowering (B) alfalfa. Volatile collections were taken at 4-hr intervals starting at 06:00 hr and ending at 18:00 hr. Each bar represents the mean ± SE for 6–8 replicates. 1 = (*E*)-2-hexenal, 2 = (*Z*)-3-hexen-1-ol, 3 = α-pinene, 4 = β-pinene, 5 = myrcene, 6 = (*Z*)-3-hexenyl acetate, 7 = (*E*)-2-hexenyl acetate, 8 = limonene, 9 = (*Z*)-ocimene, 10 = (*E*)-β-ocimene, 11 = linalool, 12 = (3*E*)-4,8-dimethyl-1,3,7-nonatriene, 13 = methyl salicylate; 14 = (*Z*)-3-hexenyl butyrate, 15 = indole, 16 = (*E*)-β-caryophyllene, 17 = (*E*, *E*)-α-farnesene, and 18 = (3*E*, 7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene.

field conditions, might necessitate a higher behavioral threshold for host finding relative to adults.

The fact that nymphs responded to all alfalfa treatments implies a lack of specificity in their response to changes in alfalfa volatiles due to phenology (i.e., flowering) or insect damage. For instance, considerable amounts of lipoxygenase products (so-called GLVs) and several monoterpenes were emitted from alfalfa

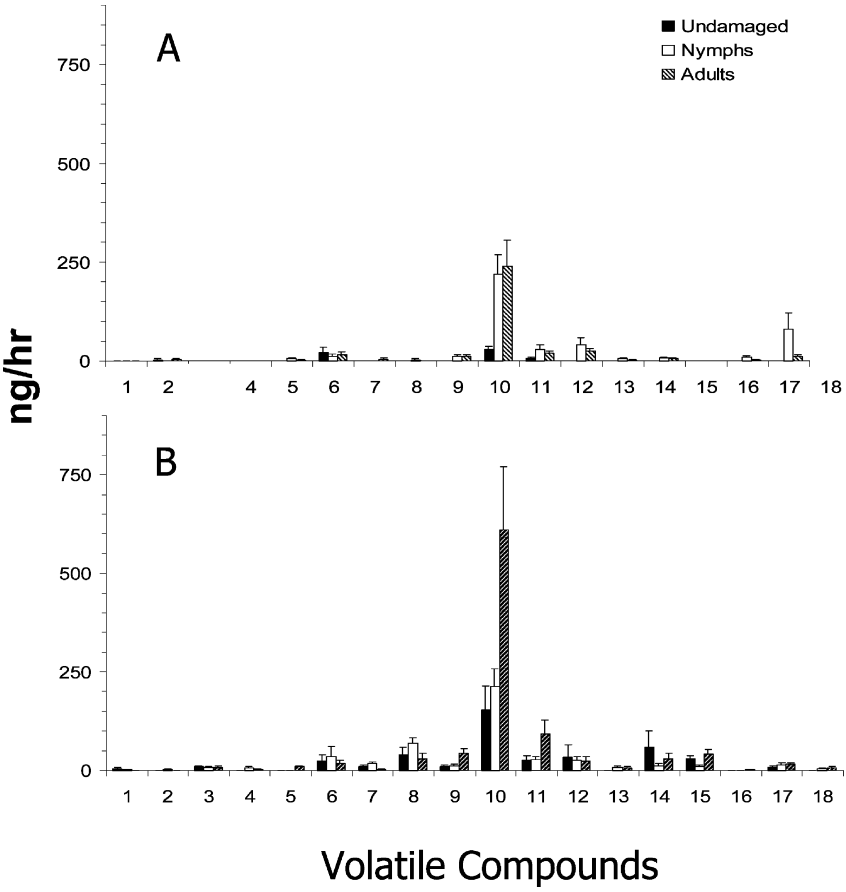

FIG. 5. Volatiles collected from the aerial portions of vegetative (A) or flowering (B) alfalfa damaged by nymphs and adults of *Lygus hesperus*. Volatiles were collected continuously for 8 hr during photophase. Each bar represents the mean ± SE for 4–7 replicates. Volatile designations are the same as described in Figure 4.

regardless of its phenology or amount of insect damage (Figures 4 and 5). GLVs and monoterpenes may serve in host location by *L. hesperus* nymphs. These compounds are produced by several other species of plants and their emissions are elevated after herbivore damage at the site of feeding and also systemically from undamaged tissues of damaged plants (Mattiacci et al., 2001). Therefore, it is likely that a polyphagous herbivore such as *L. hesperus,* that feeds on over 100 plant species, may utilize volatile cues common to a variety of plants during host location.

Female *L. hesperus* were more responsive to alfalfa headspace volatiles than males, of which the latter failed to show a preference for any alfalfa treatments (Figures 2 and 3). *Lygus hesperus* females deposit their eggs into plant tissue, so this sex-related difference in response is probably related to the female's need to find suitable substrates for oviposition. In contrast, males showed a negative response to plant odors when offered alone (without conspecifics). The strong negative response to both vegetative and flowering alfalfa is probably adaptive as it would decrease or eliminate the time males spend in areas that lack potential mates. In fact, for two other species of Miridae, *Lygus lineolaris* (Palisot de Beauvois) and *Lygocoris pabulinus* (L.), electroantennograms (EAGs) indicated that the antennal responses of female bugs were far more pronounced to plant compounds than the antennal responses of male bugs (Chinta et al., 1994; Groot et al., 1999). These studies also indicated that two ubiquitous groups of plant volatiles, GLVs (produced from the lipoxygenase pathway) and monoterpenes, caused greater responses in females as compared to male antennae. These two groups of compounds were collected from both vegetative and flowering alfalfa (Figures 4 and 5).

In addition to the differences in emissions due to plant phenology, GLVs and monoterpenes varied with time of day (Table 2; Figure 4). Similarly, Loper and Lapioli (1971) demonstrated a daily cycle for alfalfa volatile emissions that resembled a bell-shaped curve with emanations reaching a maximum 5–7 hr after the beginning of photophase. Under field conditions, Pecetti and Tava (2000) found similar patterns of emissions for the second and third flowerings of alfalfa. Our results indicated that GLVs were emitted from alfalfa soon after photophase (06:00–10:00 hr) and prior to emissions of monoterpenes that occurred primarily at midday (10:00–14:00 hr; Figure 4). Changes in the volatile composition of blends during photophase have also been reported for caterpillar-damaged maize and cotton (Turlings et al., 1998; Rodriguez-Saona et al., 2001). We also found that headspace volatile emissions for flowering alfalfa were maximal from midday to late afternoon (Figure 4). Marletto et al. (1985) demonstrated that these peaks in emissions of alfalfa volatiles at midday corresponded with increased activity in insect pollinators.

Another environmental factor that can potentially influence volatile emissions in alfalfa is insect damage (Figure 5). Damage by adults of both sexes and nymphs of *L. hesperus* induced local and systemic emissions of several GLVs and monoterpenes in cotton and maize (Rodriguez-Saona et al., 2002). Monoterpenes, such as (*E*)-β-ocimene, were also induced by *L. hesperus* feeding in alfalfa (Table 3), which may explain the greater responsiveness of females to plants that had been fed upon by conspecifics (Figure 2).

The lower-level response to alfalfa and conspecific cues exhibited by adult *L. hesperus,* in comparison to the nymphal response, suggests that for adults additional cues (i.e., visual or the combination of visual and volatile cues) could

be more important than volatile cues during the host-location process. Landis and Fox (1972) demonstrated that *L. hesperus* is strongly attracted to visual cues. Alternatively, because these insects are facultative predators, we may find that volatile cues associated with prey items are relatively important in mediating their behavior. Defensive compounds produced in the metathoracic glands (Gupta, 1961), sex pheromones, and possibly aggregation pheromones could also influence foraging decisions (Groot et al., 2001).

Finally, for adult insects that engage in flight, Y-tube olfactometers may not be the best way to examine their response to plant odors. Orientation during flight may be an integral part of the behavioral sequence that leads the adult to the host plant. Several of these possibilities are currently being investigated in our laboratory. With a better understanding of these various interactions, we hope to develop a trapping system that will be effective in monitoring both male and female *L. hesperus*. Furthermore, we know that herbivore-induced volatiles play an important role in the host-searching behavior of natural enemies (Dicke and Sabelis, 1988; Turlings et al., 1990; Agelopoulus and Keller, 1994; Powell et al., 1998); however, almost nothing is known about the cues used by natural enemies of *Lygus* spp. The fact that feeding by nymphs and adults caused increased releases of monoterpenes and sesquiterpenes, which have been found to be attractive to beneficial insects in other cropping systems (Turlings et al., 1993; Birkett et al., 2000), may enable us to monitor abundance of, or attract natural enemies of *L. hesperus*.

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DETECTION AND DISCRIMINATION OF CONSPECIFIC SCENTS BY THE ANGUID SLOW-WORM *Anguis fragilis*

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Abstract—We tested the ability of male slow-worms, *Anguis fragilis*, a limbless anguid lizard with secretive, semifossorial habits, to detect chemical associated with conspecifics by using a T-maze in the laboratory. Male slow-worms discriminated conspecific male and female scent deposits. Males selected the arm with female scent, suggesting that scent deposits may be used to locate potential mates. Also, male slow-worms did not avoid the chemicals of other males, suggesting that they are not territorial. However, males discriminated their own scent from those of other males, and spent more time exploring the arm with other male scent, which suggests that scent marks may bear information that could be used in future intrasexual social contexts. We conclude that discrimination of conspecifics based on scents may be more widespread than previously expected among lizards inhabiting visually restricted environments.

Key Words—Conspecific discrimination, self-recognition, T-maze, slow-worms, *Anguis fragilis*, reptile pheromones, substrate scent deposits, Reptilia, Anguidae.

INTRODUCTION

Pheromonal communication is used by vertebrates in many contexts, and may be especially important when visual and auditory communication is restricted or impossible (Stoddart, 1980; Wyatt, 2003). Pheromones play an important role in the intraspecific communication of many reptiles, with a variety of functions (Halpern, 1992; Mason, 1992; Cooper, 1994). For example, chemical signals may permit discrimination between males and females, or between neighbors and

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nonneighbor males, which could help to stabilize social systems (e.g., Glinski and Krekorian, 1985; Steele and Cooper, 1997; Bull et al., 2000; Aragón et al., 2001). Some reptiles may detect and follow scents passively laid on the substrate by conspecifics to form aggregations (Reinert and Zappaltori, 1998), or to locate mating partners (Ford, 1986; Bonnet et al., 1999). For example, some female snakes produce pheromone trails that guide males to their exact location during the breeding season (LeMaster et al., 2001). Pheromones have been found to not only communicate the sex, but also contain information concerning the species identification, body size, relatedness, or sexual attractiveness of the scent producer (Ford and Schofield, 1984; Aragón et al., 2000, 2001; López et al., 2002).

Pheromone-mediated location of conspecifics has been extensively documented in snakes (e.g., Ford, 1986; Greene et al., 2001; LeMaster and Mason, 2001), but is poorly documented in other groups of reptiles. A few studies have examined the use of scent trails to locate mating partners and to facilitate mate guarding in skinks (Cooper and Vitt, 1986; Olsson and Shine, 1998; Bull and Lindle, 2002). However, it might be expected that pheromone trailing is widespread among other lizards as well, because many lizards are able to detect and discriminate conspecific scent marks deposited on the substrate (e.g., Graves and Halpern, 1991; Carpenter and Duvall, 1995; Cooper et al., 1996; Aragón et al., 2001). Complex habitats also limit the visual detection of conspecifics, favoring the use of chemical cues. Therefore, the ability to use scent deposits to locate mates or avoid competitors should be especially important in semifossorial or visually restricted species, or in species inhabiting structurally complex microhabitats.

Anguids are a group of reptiles that include many species morphologically and functionally adapted to a semi-subterranean life or to complex microhabitats. For example, many species have reduced limbs, and many are limbless altogether (Wiens and Slingluff, 2001). Conspecific discrimination in anguids has not been studied, but it is known that at least some species are able to detect and discriminate prey chemicals (Cooper, 1990, 1995; Cooper and Habegger, 2000). Therefore, it is likely that anguids are also able to discriminate conspecific scents. The slow-worm *Anguis fragilis* Linnaeus 1758 is a limbless anguid lizard widely distributed throughout Europe (Dely, 1981; Cabela, 1997; Salvador, 1998). It occurs in herbaceous microhabitats with a high vegetation cover where visibility is limited, and it is usually found under logs, flat stones, or tiles. Because of its secretive, semifossorial habits, few detailed studies have been made of its ecology (Stumpel, 1985; Capula and Luiselli, 1993; Vences, 1993; Capula et al. 1996), although other aspects of its biology are well known (Dely, 1981). Slow-worms probably spend much of their active lives underground or in thick vegetation at the surface (Beebee and Griffiths, 2000) where they are active foragers (Salvador, 1998). No studies of slow-worm pheromones have been reported, but their semifossorial habits clearly limit the potential for visual detection and recognition of conspecifics. Therefore, pheromones, might be important for many aspects of slow-worms' biology.

In this study, we tested the ability of male slow-worms, *A. fragilis*, to detect and discriminate conspecific chemical stimuli by using a T-maze in the laboratory. We also tested the prediction that chemical preference levels would correlate with the nature of social relationships among individuals, also being an indirect measure of their spacing behavior (Ferkin and Seamon, 1987).

METHODS

Study Animals and Maintenance. During Spring 2003, we captured 13 adult male slow-worms, *A. fragilis* (snout-to-vent length, SVL: 172 ± 7 mm), by lifting stones in herbaceous microhabitats such as wet grassland, farmland, gardens, and woodland edges near Santiago de Compostela (A Coruña Province, NW Spain). Previously, during summer 2002, we had captured 10 adult females (SVL: 161 ± 6 mm) in the same area, that were kept isolated in captivity to ensure that they were not pregnant during this experiment.

Slow-worms were individually housed at El Ventorrillo Field Station near Navacerrada (Madrid province, central Spain), in cages (36 × 25 × 13 cm) containing sawdust and tree bark for cover. They were fed earthworms and slugs twice weekly. Prey were alive when fed to the slow-worms, and all were readily eaten. Humidity was maintained with a water spray twice a week. The photoperiod was same as that of the surrounding region, but ambient temperature was maintained at 20°C. The slow-worms were held in captivity for at least 1 month before testing to allow acclimation to laboratory conditions and the experimenter's presence. All animals were healthy during the trials, and at the end of the experiment all had maintained or increased their original body mass, and they were returned to their exact capture sites. The experiments were performed under license from the Consellería de Medioambiente de la Xunta de Galicia (Environmental Agency of the Galicia Local Government).

Experimental Design. We performed this experiment during the mating season (March to May) of slow-worms to test the ability of males to detect chemical stimuli from conspecifics, and to discriminate female scents from those of other males, and from their own scents. We used a standard T-maze constructed of clear PVC tubing with an internal diameter of 5 cm. The maze consisted of an initial base arm 50 cm in length, and two choice arms 30 cm in length and separated by an angle of 180°. Scent stimuli (sawdust from rearing cages) were placed along one of the choice arms, whereas clean sawdust was placed along the base and the control arms, with the sawdust layers being approximately 1 cm deep along all arms. Three different types of treatments were conducted for each experimental male: (1) female scent vs. blank control; (2) other male scent vs. blank control; and (3) own scent vs. blank control. The three conditions were presented in a counterbalanced sequence in a randomized block design, only one trial per day

being conducted for a given individual. In each trial, one of the three scent stimuli was randomly placed at the right or left choice arm of the maze.

The chemical stimuli consisted of sawdust that had been in the housing cages of slow-worms for at least 3 days, and that contained feces and other slow-worms' scents. Clean sawdust shavings slightly humidified with distilled water were used as blank control stimulus. The sawdust was manipulated with fresh gloves to avoid contaminating with human scents. The same sawdust was never used more than once.

Before the beginning of a test, the experimental male slow-worm was placed at the start of the base arm.The trial began when the slow-worm started to move, and lasted 60 min. Trials were conducted daily between 0800 to 1100 hr, when the slow-worms were normally more active in the field (personal observation). Environmental conditions (e.g., temperature, lighting) remained constant throughout the experiments. Between trials, we removed the sawdust, washed the T-maze with a nonodorous detergent first and then with ethanol (70%), and dried it at outdoor temperature.

Observations were always made by the same person so as to reduce variation in the data collection. For every trial, the following variables were recorded: (1) time spent in the base arm before entering the choice arms; (2) initial arm choice; (3) time spent in the choice area (i.e., the T junction between the two choice arms); (4) time spent in the control arm; (5) time spent in the experimental arm; and (6) number of changes between choice arms.

Statistical Analyses. To analyze differences between treatments in time spent in the different arms of the T-maze we used two-way repeated measures analyses of variance (ANOVA). Data were log-transformed to ensure normality (Shapiro–Wilk's test). Tests of homogeneity of variances (Levene's test) showed that in all cases variances were not significantly heterogeneous after transformation. Pairwise comparisons were planned using Tukey's honest significant difference (HSD) tests. Differences in initial arm choice between treatments were evaluated with binomial tests (Sokal and Rohlf, 1995). All statistical tests were two-tailed.

RESULTS

There were significant differences between treatments in the time spent by male slow-worms in the base arm before entering the choice arms (repeated measures ANOVA, $F = 5.20$; $df = 2, 20$; $P = 0.015$) (Table 1). The time spent in the base arm when a choice arm contained other male scent was greater than in the rest of treatments (Tukey's tests, $P < 0.05$, in all cases), but there were no differences between treatments with female or own scents ($P = 0.90$). Time spent in the choice area before the first selection did not significantly differ among treatments (repeated measures ANOVA, $F = 0.51$; $df = 2, 20$; $P = 0.60$) (Table 1).

TABLE 1. TIME SPENT BY MALE SLOW-WORMS IN EACH ARM OF A T-MAZE, AND NUMBER OF CHANGES BETWEEN ARMS, IN THREE DIFFERENT TREATMENTS WITH A BLANK CONTROL ARM AND AN EXPERIMENTAL ARM WITH CONSPECIFIC SCENT

	Treatment		
	Female scent	Other male scent	Own scent
Time in the base arm (sec)	328 ± 99	511 ± 137	234 ± 38
Time in the choice area (sec)	81 ± 18	79 ± 14	68 ± 16
Time in the control arm (sec)	443 ± 134	760 ± 211	1717 ± 209
Time in the experimental arm (sec)	2148 ± 145	1523 ± 150	353 ± 113
Relative time in the experimental arm (%)	83 ± 5	70 ± 7	17 ± 5
Changes between choice arms (n)	6 ± 1	7 ± 1	11 ± 1

A significantly greater number of male slow-worms entered the arm with female scent than the control arm in their first selection (10 vs. 1, binomial test, $P = 0.001$). In the next test, most male slow-worms chose the control arm instead of the arm with their own scent (10 vs. 1, binomial test, $P = 0.001$). In contrast, in the treatment with other male scent, males did not discriminate between the scented arm and the control arm (6 vs. 5, binomial test, $P > 0.99$).

Total time spent by male slow-worms in the experimental arm with scent differed among treatments (repeated measures ANOVA, $F = 26.19$; $df = 2, 20$; $P < 0.001$) (Table 1). Males spent significantly less time in the arm with their own scent than in the arms with scent of other male or female conspecifics (Tukey's tests, $P < 0.001$ in both cases). There was no difference in time spent in arms with treatments with other male and female scent ($P = 0.58$). Similarly, relative time spent by slow-worms in the arm with scent, in relation to the total time spent in any of the arms, differed among treatments ($F = 20.87$; $df = 2, 20$; $P < 0.001$) (Table 1). Slow-worms spent less relative time in the arm with own scent than in the arms with scents of other males or females (Tukey's tests, $P < 0.001$, in both cases), and there was no difference between relative time spent in arms with other male or female scent ($P = 0.58$).

The number of changes between the control arm and the arm with scent was different among treatments (repeated measures ANOVA, $F = 10.13$; $df = 2, 20$; $P < 0.001$) (Table 1). Slow-worms changed more often between arms in the own-scent treatment than in treatments with male or female scent (Tukey's tests, $P < 0.05$, in both cases); however, there were no significant differences between male and female scent treatments ($P = 0.77$).

DISCUSSION

Our results suggest that male slow-worms have the ability to detect and discriminate chemicals associated with conspecific males and females. These

conclusions were supported by the preferred first selection of female scent against the blank control, in contrast to the lack of differences in first selection between other male scent and a control. These results further suggest that males preferred the female stimuli. Pheromonal detection is often found in reptile species that inhabit complex habitats where the use of visual cues is limited (Olsson and Shine, 1998; LeMaster and Mason, 2001; Shine and Mason, 2001), or in semifossorial species (Cooper et al., 1994). In several reptile species, especially in snakes, males trail females' chemical cues to locate them during the breeding season (e.g., Ford, 1986; Bull and Lindle, 2002), and a similar mechanism could be used by male slow-worms to locate potential mates in their visually restricted habitat.

Male slow-worms did not avoid the sawdust with chemicals of other males. Other territorial lizards reacted to scent marks of competitor males by increasing their exploratory rate or by fleeing from the other male's chemical cues (Aragón et al., 2000, 2001, 2003). This might suggest that slow-worms are not territorial. However, male slow-worms spent more time before entering the arm with other male's scent than the arms with other scents. This suggests that males were able to detect scents of conspecific males, and that before entering the area with scent, males ensured that the other male was not around. Thus, male slow-worms would avoid the risk of a costly agonistic encounter because in the field males fight aggressively during the mating season and may deliver serious bites to their opponents (Salvador, 1998). Moreover, after entering the arm with other male stimuli, male slow-worms spent more time in it in comparison with time spent in the blank control arm. This might be explained by males' spending more time looking for additional cues to decide whether to avoid a possible agonistic interaction. Information on possible opponents could be useful later in future encounters. In this context, several studies have shown that the scent marks of male lizards can bear additional information on the sex identity, such as body size, physical condition, or potential fighting ability (Aragón et al., 2000; López et al., 2002), or even individual identity (Glinski and Krekorian, 1985; Alberts and Werner, 1993, Aragón et al., 2001), which may influence the characteristics and outcome of future agonistic encounters (López and Martín, 2002).

When slow-worms were tested with own scent vs. a blank control, they preferred the control arm, and spent less time in the arm with their own scent than in arms with other male or female chemicals. This suggests that male slow-worms are able to discriminate their own scent from those of other males. Moreover, when slow-worms were tested with their own chemicals, they changed arms more often than when tested with scent of other males or females. In animals capable of self-recognition, scent-marks are commonly used to advertise home ranges or territories (Halpin, 1986; Alberts, 1992; López et al., 1998), but self-recognition may also be useful in a dominance system where individual males overlap spatially and recognize each other, and scent marks give information on their identity, condition, or status (Aragón et al., 2001). The high population density of slow-worms in the

field (Vences, 1993), and observations of several males under the same retreats (personal observation) suggest that slow-worms might form dominance hierarchies. Scent marks could stabilize this social system by reducing the frequency of aggressive encounters between neighbor males with previously established status (Aragón et al., 2001).

The ability of male slow-worms to detect conspecific chemical stimuli, and to discriminate the sex of conspecifics and their own scent from those of other conspecifics suggests that pheromones provide an important means of communication in this species. Further studies that examine the role of conspecific chemical signals in social organization and the possible use of trailing to locate mates in this and other anguid species are needed.

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STATISTICAL PROBLEMS ENCOUNTERED IN TRAPPING STUDIES OF SCOLYTIDS AND ASSOCIATED INSECTS

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Abstract—Traps baited with semiochemicals are often used to investigate the chemical ecology of scolytids and associated insects. One statistical problem frequently encountered in these studies are treatments that catch no insects and, thus, have zero mean and variance, such as blank or control traps. A second problem is the use of multiple comparison procedures that do not control the experimentwise error rate. We conducted a literature survey to determine the frequency of these two statistical problems in *Journal of Chemical Ecology* for 1990–2002. Simulations were then used to examine the effects of these problems on the validity of multiple comparison procedures. Our results indicate that both statistical problems are common in the literature, and when combined can significantly inflate both the experimentwise and per comparison error rate for multiple comparison procedures. A possible solution to this problem is presented that involves confidence intervals for the treatment means. Options to increase the statistical power of trapping studies are also discussed.

Key Words—Scolytidae, multiple comparisons, experimentwise error rate, blank treatment, homogeneity of variances, semiochemical, pheromone.

INTRODUCTION

Bark beetles (Scolytidae) and their associates illustrate many important phenomena in chemical ecology. Studies involving these organisms have elucidated the role of aggregation pheromones and host volatiles in the colonization process of the host tree, the role of antiaggregation pheromones that apparently limit

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attack densities, and the use of kairomones by natural enemies and competitors to locate prey and resources (Borden, 1982; Wood, 1982; Smith et al., 1993; Raffa, 2001). Novel methods of control have also been developed that use these chemical signals to deflect beetle attack from host trees (Borden, 1997). Trapping experiments in the field are frequently used to study the chemical ecology of these organisms. These studies are often designed to address two basic questions: (1) which treatments (chemicals or biological material) are attractive to the insects, and (2) which treatments increase or decrease catches of insects relative to other treatments? Both questions are typically examined within the same trapping study. Completely randomized or randomized block designs are commonly used, and the resulting data are counts of the number of insects caught in each trap.

One statistical problem frequently encountered in semiochemical trapping studies are unequal variances (heteroscedasticity) among treatments, with variance-stabilizing transformations such as $\sqrt{Y}$or $\log(Y + 1)$ typically used as a remedy. In experiments that involve blank traps or other treatments that are unattractive, however, it is often the case that all the observations are zero and so have zero variance. Variance-stabilizing transformations are not useful here, because they cannot create variance where none exists. Treatments that have zero or low variance will reduce the magnitude of mean square error, which is the denominator for F tests and also a component of multiple comparison procedures used to compare treatments. Thus, blank traps and other low variance treatments could potentially affect the statistical analysis of semiochemical trapping studies by increasing apparent treatment effects.

Another problematic feature of trapping studies is the use of multiple comparison procedures that do not control the experimentwise error rate. The experimentwise error rate is defined as the probability of one or more Type I errors (spurious results) in a set of comparisons, usually all pairwise comparisons among treatments in an experiment. One commonly used procedure is Fisher's protected LSD (least significant difference) test, which is well-known to have an experimentwise error rate that increases rapidly with the number of treatments in the experiment (Hayter, 1986; Toothaker, 1993; Hsu, 1996; Westfall et al., 1999). Thus, as the number of treatments increases the probability of finding at least one significant difference by chance also increases. Some authors have argued that it is more appropriate to control the per comparison error rate (Carmer and Swanson, 1973; Carmer and Walker, 1982; Rothman, 1990; Saville, 1990; Stewart-Oaten, 1995), defined in this context as the probability of Type I error for a single pairwise comparison between treatments. Regardless of the merit of these arguments, readers of trapping studies should be aware that the two types of multiple comparison procedure have different statistical goals and are not equivalent. Methods that control the experimentwise error rate place a premium on controlling all Type I errors and so are inherently more conservative procedures. The problem with this approach is that differences that do exist may not be detected because of low

statistical power. Methods outside this category (such as Fisher's protected LSD) are more powerful but are also more likely to find spurious differences among treatments.

We had three objectives in this study. The first was to document the prevalence of the statistical problems discussed above in published studies of scolytids and their associated insects (typically competitors and predators). We conducted a literature survey to determine the frequency of blank treatments in trapping studies, the number of treatments per experiment, and the multiple comparison procedure used. The multiple comparison procedures were also classified into two groups depending on whether they control the experimentwise error rate. We confined our survey to papers on these organisms because of our familiarity with their objectives and to reduce the papers surveyed to a manageable number. The second objective was to examine the effect of blank treatments on the validity of multiple comparison procedures, in particular the experimentwise and per comparison error rates. We used simulation studies to evaluate these rates for two disparate procedures, one that controls the experimentwise error rate (Tukey's HSD or honestly significant difference) and one that does not (Fisher's protected LSD). Our third objective was to develop an alternate method of analysis that avoids these statistical problems. Various options to increase the statistical power of semiochemical trapping studies are also discussed.

METHODS AND MATERIALS

Literature Survey. We surveyed papers published in the *Journal of Chemical Ecology* involving trapping studies of scolytids and associated insects for the interval 1990–2002. For each paper, we determined whether any experiments incorporated blank traps, the average number of treatments per experiment (most papers involved several experiments), whether the analysis was parametric or nonparametric, and if parametric the multiple comparison procedure used (if any). The multiple comparison procedures we encountered were Fisher's protected LSD, simple LSD with no preliminary ANOVA, Duncan's multiple range test, Student–Newman–Keuls multiple range test, the REGW multiple range procedures in SAS (SAS Institute Inc., 2001), Tukey's HSD, Dunnett's test (which compares treatments with a control), and a Šidák adjustment for multiplicity. General descriptions of these procedures can be found in Sokal and Rohlf (1995), Hsu (1996), and Westfall et al. (1999). Of these procedures, only the last four control the experimentwise error rate (Day and Quinn, 1989; Hsu, 1996; Westfall et al., 1999).

Simulation Studies. We conducted simulations to estimate the experimentwise error rate for two different multiple comparison procedures, Fisher's protected LSD and Tukey's HSD, in randomized block experiments involving a blank trap treatment. These procedures were chosen because they span the range of control

of the experimentwise error rate (none to strong) and are easy to simulate. Fisher's protected LSD is also one of the most commonly used multiple comparison procedures in the literature. Blank traps were assumed to catch zero insects and thus have zero variance, as occurs in many pheromone trapping studies (e.g., Herms et al., 1991; Miller et al., 1997; Zhou et al., 2001). All other treatments were defined to have the same mean and variance, implying there are no real treatment effects in the experiment beyond the blank treatment. We were interested in determining the error rate for all pairwise comparisons among these other treatments, excluding any comparisons involving blank traps versus other treatments. These were excluded because the null hypothesis was always false in this case (blank traps by assumption catch fewer insects than any other treatment). We also examined how the experimentwise error rate varied with the number of treatments in the simulations.

The parameter values in the simulations were based on trapping experiments (seven total) involving the scolytid predator *Thanasimus dubius* (Coleoptera: Cleridae) (J. D. Reeve, B. L. Strom, L. Rieske-Kinney, and B. D. Ayres, unpublished data). The treatments involved various bark beetle pheromones and tree volatiles, and typically captured 10–100 adult predators per trap during the course of the experiment, except for blank traps that caught virtually no insects. The data were transformed before analysis using the log transformation $\log_{10}(Y + 1)$. On this scale of measurement, we observed a mean square error $\sigma^2 \approx 0.06$, after excluding unattractive treatments from the analysis. The variance due to the block effect was considerably smaller than σ^2, and based on these studies we chose two different values for the simulations, $\sigma_B^2 = 0$ or 0.03. In terms of standard deviations, these values correspond to $\sigma = 0.245$ and $\sigma_B = 0$ or 0.173. Observations for the simulations were generated using a standard statistical model for randomized block designs:

$$Y_{ij} = \mu + \alpha_i + B_j + \varepsilon_{ij},$$

where μ is the grand mean, α_i is the effect of ith treatment, B_j is a normal random variable with mean zero and variance σ_B^2, and ε_{ij} is normal with mean zero and variance σ^2. This model specifies that treatments are fixed while blocks are random effects. Block was considered a random effect because the blocks used in semiochemical studies are typically a sample of possible study sites. We used $\mu = 1.1$ as the grand mean in the simulations, corresponding to the average number of insects captured in our experiments (on a log scale). Treatment effects were assumed to be absent, implying that $\alpha_i = 0$ for all i. Observations for the blank treatment were generated by forcing Y_{ij} to zero for that treatment across all blocks. A total of 10 blocks was used in each replicate simulation. We also varied the number of treatments in the experiment, ranging from 3 to 10 treatments. In a second set of simulations, we added another unattractive treatment to the experiment, so that there were two with zero mean and variance (a fairly common

occurrence in trapping studies). We note that the F test for the treatment effect was always significant using these parameter values, and the block effect frequently so when $\sigma_B^2 = 0.03$. The simulations were programmed using the statistical language R 1.7.0 (The R Development Core Team, 2003).

Each simulated data set was analyzed using ANOVA for randomized block designs followed by multiple comparisons using Fisher's protected LSD or Tukey's HSD. For Fisher's protected LSD, we used $\alpha = 0.05$ as the significance level for both the ANOVA and pairwise comparisons, whereas for Tukey's HSD we used an experimentwise error rate of 0.05. For each data set, the program counted the number of significant pairwise comparisons, excluding those involving the blank traps. The experimentwise error rate was estimated as the fraction of data sets having at least one significant comparison (a Type I error) in 5000 replicate simulations. We also computed the experimentwise error rate assuming the blank treatment had the same variance as other treatments, for comparison with the rates obtained with zero variance (the simulations). Here, we were able to directly calculate the error rate using the Studentized Range distribution, and in the case of Fisher's protected LSD, by assuming that the treatment F test was significant, as would be expected given the hypothesized treatment means (Hayter, 1986; Hsu, 1996). This error rate can be regarded as a predicted error rate for Fisher's protected LSD and Tukey's HSD without the zero variance problem caused by blank traps.

Using the same simulations, we also estimated the per comparison error rate for Fisher's protected LSD. This was estimated as the fraction of data sets having a significant comparison between an arbitrary pair of treatments, again excluding the blank treatment from consideration.

RESULTS

Literature Survey. The survey indicates that experiments involving blank treatments are common in pheromone trapping studies of scolytids and associates. Of the 50 papers that met our criteria, 84% (42 of 50) of the papers contained a blank or control treatment, which typically caught few or no insects. Parametric procedures were used in 78% of the papers (39 of 50), with 92% of these (36 of 39) using various multiple comparison procedures to test for differences among treatments. Averaging across all papers, the mean number of treatments per experiment was 5.66 (SD = 3.03, range 2–20). Of the papers using multiple comparison procedures, 56% (20 of 36) used methods that do not control the experimentwise error rate (Table 1). The most commonly used methods were LSD (either Fisher's protected LSD or simple LSD) and the REGW procedures of SAS (SAS Institute Inc., 2001). Of those papers using multiple comparisons, 42% (15 of 36) used methods that failed to control the experimentwise error rate and also had blank treatments, meaning they had both of these statistical problems.

TABLE 1. FREQUENCY OF VARIOUS MULTIPLE COMPARISON PROCEDURES IN JOURNAL OF CHEMICAL ECOLOGY PAPERS FOR 1990–2002

Procedure	Number of papers	Control experimentwise error rate?
Duncan's multiple range	6	No
LSD	10	No
Student–Newman–Keuls	4	No
Dunnett's test	1	Yes
REGW	12	Yes
Šidák	1	Yes
Tukey's HSD	2	Yes
Total	36	

Note. Procedures were classified as controlling the experimentwise error rate based on Hsu (1996) and Westfall et al. (1999).

Simulation Studies. The experimentwise error rates for Fisher's protected LSD and Tukey's HSD are shown in Figure 1, for simulations involving a single blank treatment with zero mean and variance. The experimentwise error rate for the protected LSD increases rapidly with the number of treatments and a blank treatment increases the rate even further, as compared to the predicted error rate for this procedure (Figure 1A). However, the effect of the blank treatment was less in the presence of a block effect ($\sigma_B^2 = 0.03$). This likely occurs because the blank treatment generates a Treatment × Block interaction in this situation (see Discussion). The experimentwise error rate for Tukey's HSD is not influenced by the number of treatments, as would be expected, and is somewhat elevated by the blank treatment although the overall rate remains low. As with the protected LSD, a block effect reduces the influence of the blank treatment on the experimentwise error rate. The addition of a second unattractive treatment increases the experimentwise error rate even further, especially in comparison to the predicted error rate for each procedure (Figure 2).

We also examined the per comparison error rate for Fisher's protected LSD, because this is the error rate nominally controlled by this procedure. The simulations indicate that the per comparison rate is elevated by a blank treatment above the 0.05 level, especially if there is a second unattractive treatment in the experiment (Figure 3). The effect on the per comparison rate diminishes as the number of treatments increases, presumably because a blank treatment has less effect on mean square error when there are more treatments. A strong block effect ($\sigma_B^2 = 0.03$) also reduces the effect of the blank treatment on the per comparison error rate.

Additional simulations (not shown) using different values of σ^2 and the number of blocks yielded virtually identical results to those in Figures 1–3, suggesting these patterns are independent of the actual parameter values chosen.

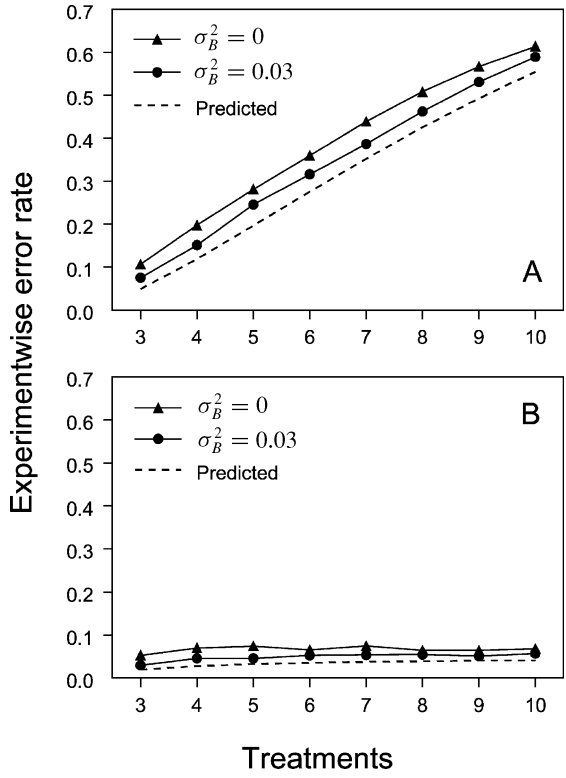

FIG. 1. Experimentwise error rate for Fisher's protected LSD (A) and Tukey's HSD (B) in simulated experiments incorporating a blank treatment with zero mean and variance, and no other treatment effects. Error rates are plotted as a function of the number of treatments and the presence or absence of a block effect ($\sigma_B^2 = 0$ vs. 0.03). The dashed line is the predicted error rate when the blank treatment has the same variance as other treatments, calculated using the Studentized Range distribution.

DISCUSSION

The literature survey indicates that blank, unattractive treatments are a common feature of trapping studies, as are multiple comparison procedures that do not control the experimentwise error rate. The simulations involving Fisher's protected LSD suggest the combination of these two features can lead to high experimentwise error rates. It can also substantially elevate the per comparison error rate, the only rate really controlled by this procedure. Fisher's protected LSD thus seems less than ideal for analyzing data of this type, especially when blank traps are present. The experimentwise error rate for Tukey's HSD was also increased by

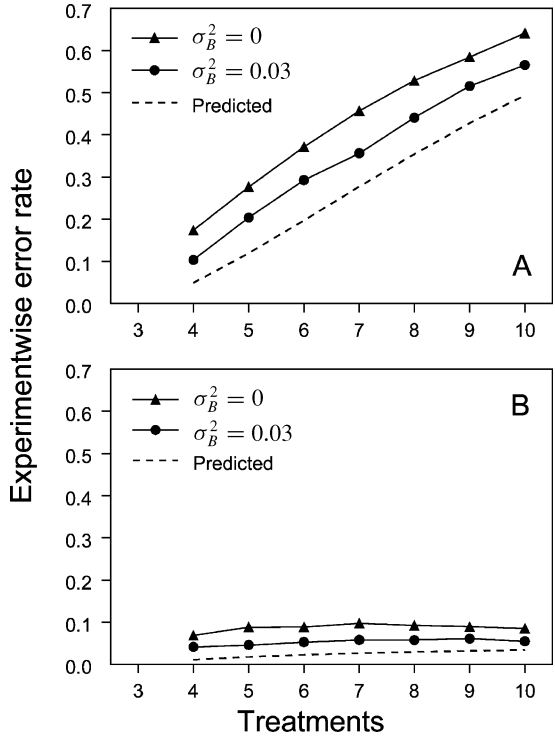

FIG. 2. Experimentwise error rate for Fisher's protected LSD (A) and Tukey's HSD (B) in simulated experiments incorporating two treatments with zero mean and variance, and no other treatment effects. For further details see Fig. 1.

unattractive treatments, but was not a function of the number of treatments and remained relatively low in any event. This result suggests that authors interested in controlling the experimentwise error rate could use a procedure such as Tukey's HSD even with blank treatments in the experiment. The mechanism underlying these results is the reduction in mean square error caused by zero variance treatments in the data, causing it to underestimate the variability among observations in the other treatments in the design. Many other multiple comparison procedures use mean square error in their formulation and we would expect their error rates to also be inflated, although the rates for procedures that control the experimentwise error rate should be lower.

An interesting finding in the simulations was the interplay between the block effect and the per comparison and experimentwise error rates. The presence of a block effect reduces these error rates apparently because it generates a Treatment $\times$ Block interaction in the data. The source of this interaction is the fact that blank

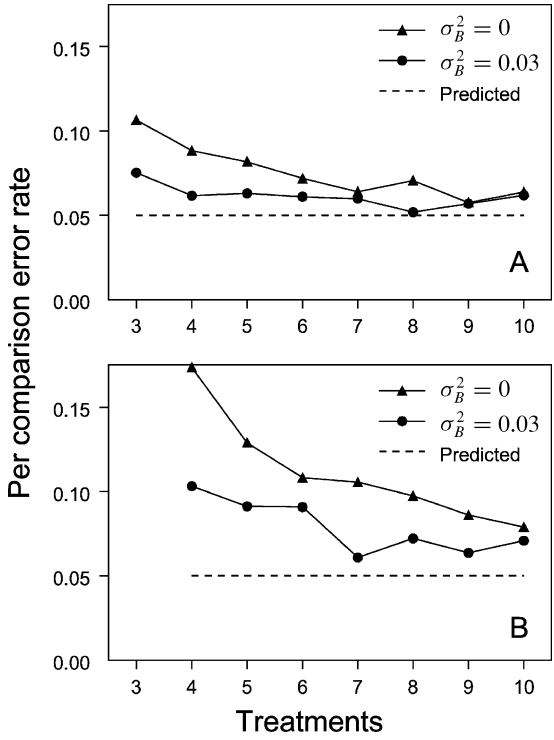

FIG. 3. Per comparison error rate for Fisher's protected LSD in simulated experiments incorporating one (A) or two (B) treatments with zero mean and variance, and no other treatment effects. The dashed line is the predicted per comparison error rate when the blank treatment has the same variance as other treatments.

treatments always have zero mean regardless of any differences in block means. The statistical model for randomized block designs has no interaction term to absorb this source of variation, so instead it inflates the value of mean square error, partially countering the deflating effect of the blank treatment. This result implies that the per comparison and experimentwise error rates are to some extent indeterminate when blank treatments are included in the analysis, because they apparently depend on the magnitude of the block effect.

A possible solution to these statistical problems would be to remove the blank treatment (and possibly other zero variance treatments) from the analysis. This would ensure that error rates are near their specified values, whatever multiple comparison procedure is used. The elimination of unattractive treatments from the data would also facilitate analysis using generalized linear mixed models, an extension of mixed models to Poisson or other discrete distributions (McCulloch

and Searle, 2001). In our experience, the software developed for these models can have numerical problems with concentrations of zeros.

Removal of the blank treatment does present a problem in evaluating which treatments are attractive to insects (a common objective of semiochemical trapping studies) because it leaves no standard with which to compare the remaining treatments. One way of dealing with this problem is to specify some minimum level of attraction believed to have biological relevance and compare the treatments to this level. For example, an investigator could specify that a treatment must exceed an average of one insect per trap during the course of the experiment to be considered attractive. Confidence intervals for the treatment means could be used to make this decision more quantitative and are more informative for this reason than the usual standard errors. If we are only interested in treatments that exceed a specified level, then one-sided confidence intervals may be appropriate. If the confidence interval boundary exceeds the specified level of attraction, this actually constitutes an α-level test of H_0: $\mu = \mu_0$ versus A: $\mu > \mu_0$, where μ_0 is the specified level of attraction and $100(1 - \alpha)\%$ is the confidence level of the interval. We also suggest applying a Bonferroni correction to the value of α for the confidence intervals because we are constructing multiple intervals (one for each treatment). Finally, if transformations are used in the analysis it may be necessary to back-transform the confidence intervals to the original scale of measurement for comparison with μ_0 (or transform μ_0 itself). A sample SAS program and data set that illustrates these calculations is given in Appendix A. The sample data are trap catches of *T. dubius* from a randomized block experiment with six blocks and five treatments, including a blank treatment that caught no insects.

The program in Appendix A uses a standard mixed model for randomized block designs that assumes the data are normally distributed. Semiochemical trapping data are typically in the form of counts, however, and so this methodology may not be ideal, especially if a low number of insects are trapped. Generalized linear mixed models provide an alternative method of analysis for data of this type, and are implemented in SAS by the GLIMMIX macro (Littell et al., 1996; SAS Institute Inc., 2001). Appendix B lists a sample SAS program that uses this method to find confidence intervals for the treatment means. The model assumes the data are Poisson in distribution but also allows for over- or underdispersion in the observations. One and two-sided intervals for these data are shown in Figure 4, along with a reference line suitable for testing H_0: $\mu = 1$ versus A: $\mu > 1$, where μ is the mean number captured. We would reject this null hypothesis for any treatment whose one-sided lower confidence interval lies above this line, and accept it otherwise.

So far, we have dealt with problems of Type I error without addressing issues of statistical power. *Power* is defined as the probability of rejecting a null hypothesis when it is false and some alternative is true. Multiple comparison procedures involve the testing of multiple hypotheses, however, and several definitions of power have been developed for this situation (see Westfall et al.,

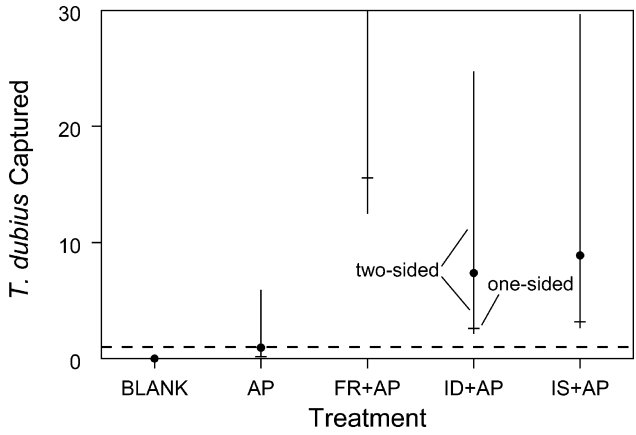

FIG. 4. One- and two-sided confidence intervals obtained using the sample data and program listed in Appendix B. The observations were obtained in a trapping experiment involving five semiochemical treatments (BLANK = blank trap, AP = α-pinene, FR + AP = frontalin + α-pinene, ID + AP = ipsdienol + α-pinene, IS + AP = ipsenol + α-pinene). The graph is truncated from above to show the confidence intervals for the less attractive treatments in more detail. A reference line for testing H_0: $\mu = 1$ vs. A: $\mu > 1$ is also shown.

1999). The easiest to calculate is individual power, defined as the probability of finding a significant difference between a given pair of treatments that differ by amount δ. We conducted a power analysis to examine how individual power varies with δ and the number of treatments and blocks in a randomized block design, using Tukey's HSD as the multiple comparison procedure. Power is also a function of mean square error or σ^2, and we used the same value as obtained in our trapping study of *T. dubius* ($\sigma^2 = 0.06$) after log transformation of the data (see Methods and Materials for further details). We used $\delta = 0.3$, 0.5, and 0.7 and five versus 10 treatments in the analysis. These values of δ correspond to approximately two-, three-, and five-fold differences in the treatment means on the original scale of measurement (before transformation). The results of the power analysis are shown in Figure 5. Power values of 0.8 or greater are typically regarded as sufficient (Cohen, 1988), and by this criterion we would have adequate power to detect three or five-fold differences between a pair of treatments using just eight blocks, although two-fold differences would require a much larger number of of blocks. This not a prohibitively large number of traps, and demonstrates that it is possible to have adequate power as well as control of the experimentwise error rate.

There are also ways of increasing power involving changes in only the experimental design, rather than increasing the number of traps deployed. If an experiment can be constructed as a comparison of a control treatment with other treatments, then one could use Dunnett's test as the multiple comparison procedure.

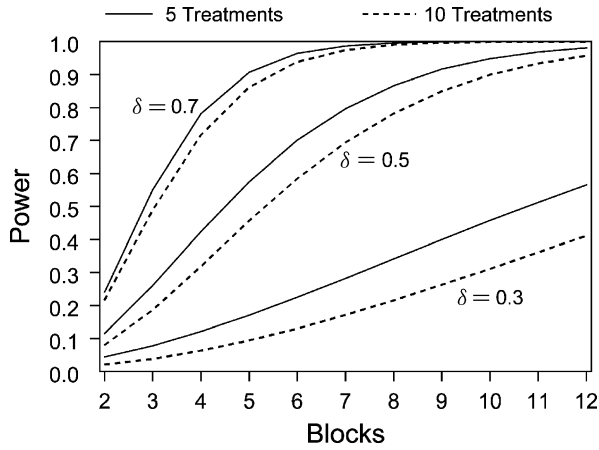

FIG. 5. An analysis of individual power for Tukey's HSD as a function of the number of blocks and treatments in the experimental design. Here δ is the difference between a given pair of treatments on a log (base 10) scale. See text for further details.

This procedure only involves pairwise comparisons of other treatments with the control, rather than all possible pairwise comparisons, and because it involves fewer comparisons is a more powerful test for experiments of this type (Hsu, 1996). We also suggest that investigators carefully consider the utility of including blank traps in every experiment for a particular insect species.

For experiments where the response to blank traps in unknown this treatment is a necessity. Blank traps will also be required in dose-response or other experiments in which it can reasonably be expected that some treatments may be negligibly attractive. In such experiments, blanks serve as a critical reference point, particularly under changing field conditions. However, in experiments comparing semiochemicals that are known to be attractive, blank traps may be wasteful particularly if previous studies have shown that they are unattractive. Resources devoted to implementing a blank treatment may be better utilized in increasing the sample size of other treatments, in particular the number of blocks for randomized block designs.

APPENDIX A

Sample SAS program and data set to find confidence intervals for the treatment means in a randomized block design using PROC MIXED in SAS (SAS Institute, 2001). The program first deletes the blank treatment observations, so that there are four remaining treatments in the analysis (see Discussion), and then log-transforms the observations. The confidence intervals for the treatment

means are generated by the lsmeans statement. To obtain a two-sided interval with a Bonferroni correction, we used $\alpha = 0.05/4 = 0.0125$ in the lsmeans statement. One-sided lower confidence intervals can be obtained by specifying twice this value ($\alpha = 0.025$) and using just the lower boundary. The intervals are then back-transformed to the original scale of measurement.

```
data traps;
      input block treat $ count;
      if treat="BLANK" then delete;
      logcount = log10(count+1);
      datalines;
1     AP      4
1     BLANK   0
1     FRAP   79
1     IDAP    7
1     ISAP   10
2     AP      1
2     BLANK   0
2     FRAP  124
2     IDAP   13
2     ISAP   20
3     AP      0
3     BLANK   0
3     FRAP   14
3     IDAP    .
3     ISAP    2
4     AP      0
4     BLANK   0
4     FRAP   15
4     IDAP   11
4     ISAP    7
5     AP      0
5     BLANK   0
5     FRAP   29
5     IDAP    7
5     ISAP    7
6     AP      2
6     BLANK   0
6     FRAP   70
6     IDAP   14
6     ISAP   20
;
run;
```

```
proc mixed data=traps;
      class treat block;
      model logcount = treat / ddfm=kr;
      random block;
      * Confidence intervals for treatment means;
      lsmeans treat / cl alpha=0.0125;
      ods output LSMeans=intervals;
run;
data intervals;
      set intervals;
      * Back-transform confidence intervals;
      Mu = 10**(Estimate)-1;
      LowerCL = 10**(lower)-1;
      UpperCL = 10**(upper)-1;
run;
proc print data=intervals;
run;
```

APPENDIX B

Sample SAS program to find confidence intervals for the treatment means using the GLIMMIX macro in SAS (Littell et al., 1996, SAS Institute, 2001). The macro fits a generalized linear mixed model to the data, assuming they are Poisson in distribution and using a log link function to connect the Poisson means to the mixed model. The confidence intervals for the treatment means are generated by the lsmeans statement. One- and two-sided intervals are obtained using the same values of α as in Appendix A. The intervals are automatically back-transformed to the original scale of measurement by the macro.

```
data traps;
      input block treat $ count;
      if treat="BLANK" then delete;
      datalines;
1     AP      4

(data as in Appendix A)

6     ISAP   20
;
run;
%include "glmm800.sas";
%glimmix(data=traps,
      procopt=method=reml,
      stmts=%str(
```

```
        class treat block;
        model count = treat / ddfm=kr;
        random block;
        lsmeans treat / cl alpha=0.0125;),
        error=poisson,
        link=log);
run;
```

Acknowledgments—This study was supported in part by a cooperative agreement with SRS-4501, Southern Research Station, USDA Forest Service. We especially thank two reviewers of the manuscript and the participants of the workshop "Methodological Problems and Solutions in Bark Beetle Semiochemical Research" at the 2003 Southern Forest Insect Work Conference, New Orleans, Louisiana.

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BENZOIC ACID DERIVATIVES IN A HYPOGASTRURID COLLEMBOLAN: TEMPERATURE-DEPENDENT FORMATION AND BIOLOGICAL SIGNIFICANCE AS DETERRENTS[1]

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Abstract—Two phenolic acids were identified in the collembolan *Ceratophysella denticulata*: 3-hydroxy-4,5 dimethoxy benzoic acid and 4-hydroxy-3,5-dimethoxybenzoic acid (syringic acid). These are localized on or in the integument of the springtail, in field-collected animals, in a ratio of 47:100 (v/v). Springtails kept under different temperature regimes showed differences in production and ratio of the benzoic acid derivatives. At 20°C, *C. denticulata* produced only syringic acid, whereas at 10°C both isomers in a ratio of 100:61 (v/v) were detected. Bioassays with *C. denticulata* as well as with the specialized collembolan predator *Stenus comma* (Staphylinidae) were carried out. Staphylinid beetles topically treated with the acids try to clean their mouthparts by rubbing them on the ground significantly more often than do control beetles. Both compounds individually and as a natural mixture have deterrent effects towards the predator *S. comma*.

Key Words—Chemical defense, Collembola, *Ceratophysella denticulata*, 3-hydroxy-4,5-dimethoxybenzoic acid, 4-hydroxy-3,5-dimethoxybenzoic acid, deterrent, body surface, cleansing behavior, predator, temperature dependency.

[1]Dedicated to Prof. Dr. Axel Zeeck (Institut für Organische und Biomolekulare Chemie, Universität Göttingen, Germany) on the occasion of his 65th birthday.

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INTRODUCTION

Chemical defense and chemical communication plays an important role in hemi- and euedaphic collembolans. Because their furca often is reduced or absent, they are unable to escape predators by jumping (Hopkin, 1997). Release of irritant, sticky chemicals mostly by reflex bleeding is used as a defense mechanism. This behavior is known from Onychiuridae (Paclt, 1956; Dettner et al., 1996). Podurid collembola are often gregarious. Therefore, alarm and aggregation pheromones are important for them (Joosse and Koelman, 1979; Purrington et al., 1991; Messer et al., 1999; Manica et al., 2001).

A predator specialized to catch Collembola is the staphylinid beetle *Stenus comma* LeConte (Coleoptera: Staphylinidae). This beetle possesses a labium with a sticky tip, which can be protruded for prey capture in less than 3 msec. The whole sequence of movements for attack is completed within 30–40 msec (Bauer and Pfeiffer, 1991; Betz, 1998). After contact with collembolans, like *Podura aquatica* L. (Collembola: Poduridae), that are reported to be toxic or unpalatable to arthropod predators, the beetles spit them out immediately and clean their mouthparts by rubbing them on the ground (Bauer and Pfeiffer, 1991). Some ant and mite species are known to be deterred from eating hypogastrurid Collembola (Panic, 1963; Wilson, 1950; Vannier, 1971; Harris and Usher, 1976), but until now nothing has been published about the chemical nature of the hemolymph contents of hypogastrurid Collembola and its biological function. We report on the identification and localization of two biologically active components in *Ceratophysella denticulata* (Bagnall) (Collembola: Hypogastruridae) and show the temperature dependence of their production. The significance of these chemical constituents as deterrents towards the staphylinid *S. comma* is considered.

METHODS AND MATERIALS

Collection and Cultivation. The collembolan species *C. denticulata* was collected in November in a spruce forest near Bad Steben, Bavaria (50°20′N, 11°40′ E). Collembolans were extracted from the collected soil using a Tullgren funnel. For breeding, the animals were kept in glass vessels (100 × 80 × 70 mm) containing a moist layer of plaster of Paris with charcoal. Dry baker's yeast (Windhager) was offered as a food source. The staphylinid beetles *S. comma* were caught in a sand pit close to ponds near Bayreuth, northern Bavaria (50° 00′ N, 11° 40′ E), and kept individually in vessels with humid sand. They were fed with the collembolans *Folsomia candida* and *Sinella coeca,* from a laboratory culture, *ad libitum*. Both, Collembola and beetles, were cultivated in an environmental chamber at 20°C with a photoperiod of 12L:12D. A second culture of *C. denticulata* was kept at 10°C with a photoperiod of 10L:14D.

Extraction. Samples were prepared from one to four individuals of *C. denticulata* immersed in 10 μl of ethyl acetate (SupraSolv, Merck) for 30 min at room temperature. These samples were either analyzed directly or derivatized with N-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA, Macherey-Nagel) at 60°C for one hr. The solvent and excess MSTFA were evaporated under a gentle nitrogen stream, and the sample was redissolved in 10 μl of ethyl acetate.

Gas Chromatographic–Mass Spectrometric (GC-MS) Analyses. Samples were immediately analyzed in a GCQ gas chromatograph (Finnigan MAT) coupled with a GCQ MS detector (carrier gas: Helium, 40 cm/sec; injector temperature: 230°C; fused silica capillary column: SGE HT5, length 25 m, film thickness 0.1 μm, I.D. 0.22 mm; temperature program: 2 min isotherm 60°C, heating rate 10°C/min, final temperature 300°C) with splitless injection. The mass spectrometer was operated in scan mode detecting the ions generated by electron impact ionization (70 eV) at the ion source temperature of 150°C. Further GC-MS analyses were performed with an Hewlett-Packard model 5973 mass-selective detector connected to an Hewlett-Packard model 6890 gas chromatograph equipped with a BPX-5 (SGE) fused-silica column, length 25 m, film thickness 0.1 μm, I.D. 0.22 mm; temperature program: 2 min at 60°C, then with 10°C/min to 300°C with splitless injector. The compounds were identified by comparison with mass spectra and retention times of authentic reference samples. For quantification with GC-MS, external standards were injected (3-hydroxy-4,5-dimethoxybenzoic acid, Sigma, 97%; 4-hydroxy-3,5-dimethoxybenzoic acid, Merck, 96%). Analyses of field-caught animals, and animals reared at 20 and 10°C were carried out.

Localization of aromatic acids was tested by washing the whole animal surface. The Collembola were washed twice for 1 min in 10 μl of ethyl acetate each and both solvent extracts were pooled. Afterwards, a total extraction of the washed animals was carried out by crushing them.

Bioassays. Behavioral assays for the intraspecific response of *C. denticulata* in the T-tube, similar to the method of Messer et al. (1999), were employed using animals from the 20°C-breeding. Aromatic acids were dissolved in 0.5 M dimethylsulfoxide (DMSO) prepared with deionized water. The concentrations presented to *C. denticulata* were the maximum amount found in one animal (73 ng 3-hydroxy-4,5-dimethoxybenzoic acid, 190 ng 4-hydroxy-3,5-dimethoxybenzoic acid). Each compound was tested individually and as a nature-identical mixture (v/v 47:100) 20 times.

Bioassays with the specialized predator of Collembola, *S. comma*, took place in a Petri dish (diam 34 mm) with a moistened filter paper circle. The latter was changed after every animal. Exactly 0.5 μl of the test fluid in DMSO was topically applied to the mandibles of a narcotized (carbon dioxide) staphylinid beetle to test the biological significance. The beetle (10 specimens per tested sample) was observed singly in a light-exposed arena for 10 min after awakening from the narcocis using a video camera (JVC, BY-10E) and a recorder (Blaupunkt,

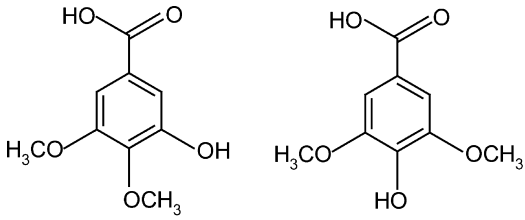
RTV-920 HIFI). The absolute duration of behavioral reactions like cleansing their mouth organs and rubbing them on the ground was taken. The concentrations were the same as in tests with *C. denticulata*.

Statistics. All statistical analyses were performed using STATISTICA 5.5 (StatSoft). The results were analyzed for significant differences between the control DMSO and the tested compounds using the Mann–Whitney *U*-test.

RESULTS

Identification and Quantification of Phenolic Compounds. Whereas the main components in field animals of *C. denticulata* were some fatty acids and squalene, two minor components were also present (Figure 1). The minor components were identified by comparison with library spectra (NIST, WILEY7) and gas chromatographic retention times with authentic samples as 3-hydroxy-4,5-dimethoxybenzoic acid and 4-hydroxy-3,5-dimethoxybenzoic acid (syringic acid). Because of the large tailing, both compounds were silylated using MSTFA. The gas chromatogram in Figure 2 shows the asymmetric isomer 3-hydroxy-4,5-dimethoxybenzoic acid as the first eluting peak, followed 0.46 min later by the symmetric 4-hydroxy-3,5-dimethoxybenzoic acid. The ratio of the two compounds in field animals was 47:100 (asymmetric/symmetric). The mass spectra of both trimethylsilyl (TMS) derivatives are similar in fragmentation pattern, but differ in the intensities of the main fragments (Figure 3). Their unusual appearance is typical for TMS-ethers of *ortho*-methoxy phenols (Krauss et al., 1985). The three characteristic ions at $m/z = 327$, 312, and 297 are formed most probably by loss of CH_3, C_2H_6, and C_3H_9 (Krauss et al., 1985).

Animals from our breeding at 20°C and 10°C yielded different results. Syringic acid as a single compound was detected in animals kept at 20°C, but both benzoic acid derivatives were established in *C. denticulata* cultivated at 10°C (Figure 2). The ratio of the isomers was 100:61 (asymmetric/symmetric).



1 **2**

FIG. 1. Benzoic acid derivatives present in the collembolan *Ceratophysella denticulata*: 3-hydroxy-4,5-dimethoxybenzoic acid (**1**) and 4-hydroxy-3,5-dimethoxybenzoic acid (**2**).

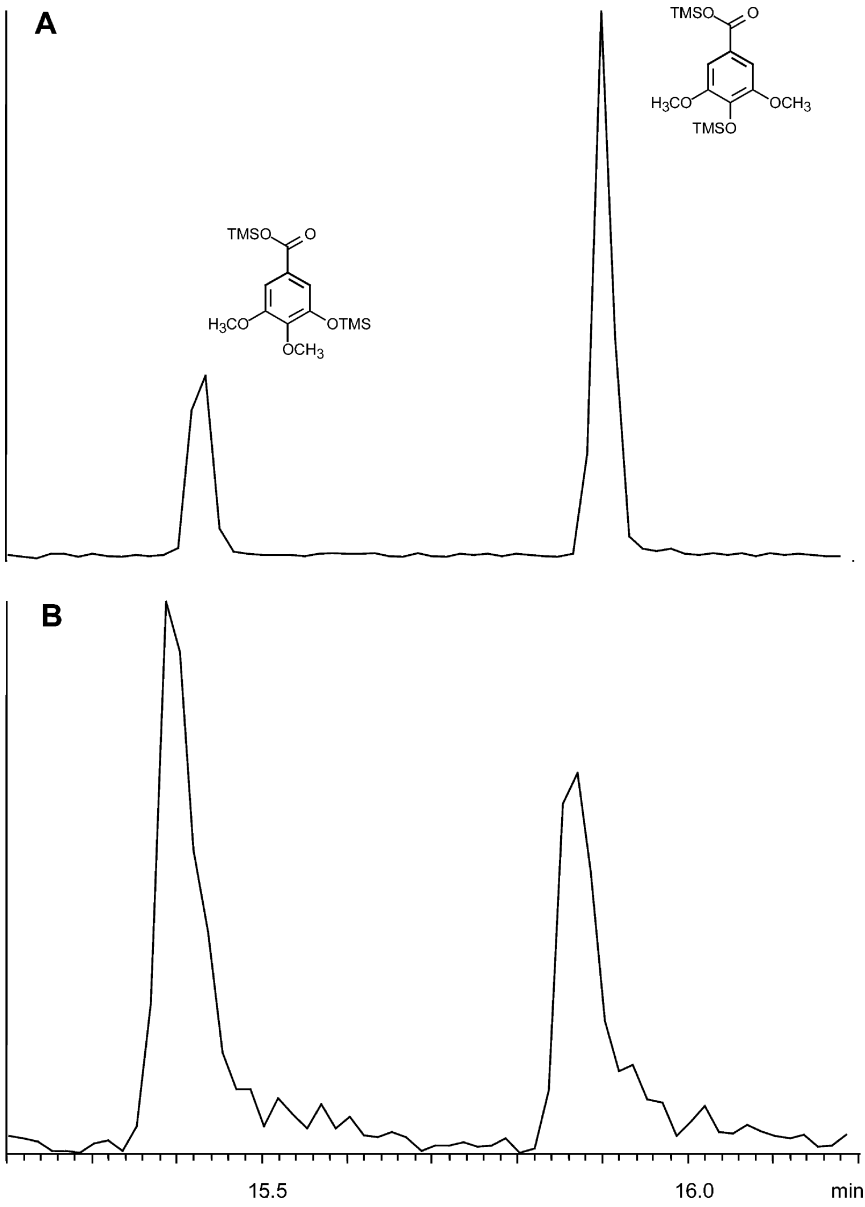


FIG. 2. Gas chromatogram of a whole extract of field animals (**A**) and animals kept at 10°C (**B**) from *Ceratophysella denticulata* derivated with MSTFA, 25-m HT5, temperature programed from 60°C (2 min isotherm) to 300°C at 10°C/min, 60 sec splitless. Slightly different retention times depend on the age of the column.

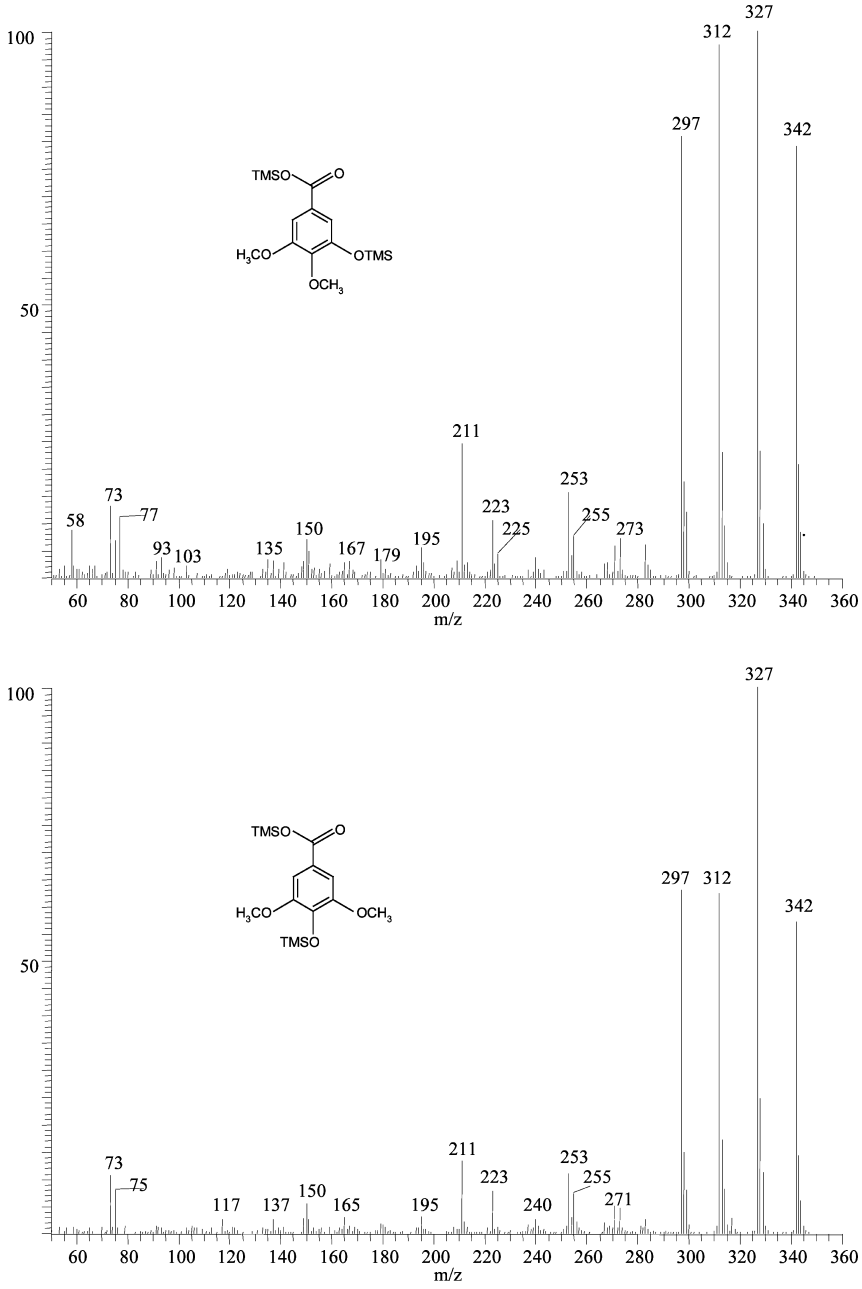


FIG. 3. Mass spectra of the benzoic acid derivatives after silylation with MSTFA.

The highest amount of the asymmetric compound found in the animal extract was 73 ng, whereas for the syringic acid this was 190 ng. These values are only approximate values because of the lability of the derivatized phenolic acids only a few samples ($N = 4$) could be quantified exactly. Analysis of the food source, baker's yeast, showed that the free acids are not present.

Localization in the Animal. The two isomers are localized on the body surface of *C. denticulata*. In surface washes, both acids were detected. The total extraction of animals after surface washes was negative; only traces of the compounds were found. Thus, the compounds are localized on or in the integument and not in the hemolymph.

Behavioral Responses. Behavioral tests with predators and conspecifics were performed. Tests within the T-tube with *C. denticulata* showing their intraspecific reaction towards the phenolic acids were not all significant (Table 1). The collembolans neither wandered significantly more often towards the offered substances nor away from them. The predator *S. comma* showed two behavioral responses after topical application: cleaning of mouthparts with its frontlegs and rubbing them on the ground. The latter was often accompanied by retching and vomitting. The duration of these responses are shown in Table 2. While "mouthpart cleaning" was not significant after application of the acids, the time *S. comma* spent rubbing its mouthparts on the ground was significantly higher in all cases than the control treatment. These results are presented in Figure 4. The largest effect was found with syringic acid alone ($P = 0.002$). The mixture caused nearly the same reaction as the asymmetric acid ($P = 0.013$).

TABLE 1. RESULTS OF MANN–WHITNEY *U*-TEST FOR ALL COMPOUNDS TESTED IN BIOASSAYS FOR DETERRENCY USING *Stenus comma* AND IN ASSAYS FOR INTRASPECIFIC BEHAVIOR

Compound/reaction[a]	Interaction[b]	*Z*	*P*
A/C	IRS	−0.378	0.705
S/C	IRS	−0.265	0.791
Mix/C	IRS	−0.151	0.880
A/R	IRS	−2.268	0.023
S/R	IRS	−3.024	0.002
Mix/R	IRS	−2.495	0.013
A	IAS	−1.096	0.273
S	IAS	−0.190	0.850
Mix	IAS	−1.327	0.185

[a] A = asymmetric form, 3-hydroxy-4,5-dimethoxybenzoic acid; S = symmetric form, 4-hydroxy-3,5-dimethoxybenzoic acid; Mix = mixture of both, v/v 47:100; C = clean, *S. comma* cleaning its mouthparts; R = rub, *S. comma* rubbing its mouthparts on the ground.

[b] IRS = interspecific interaction of the compounds tested with *S. comma*; IAS = intraspecific interaction of the compounds tested with *C. denticulata* in the T-tube.

TABLE 2. BEHAVIORAL RESPONSES OF THE COLLEMBOLAN PREDATOR *Stenus comma* TOPICALLY TREATED WITH BENZOIC ACID DERIVATIVES[a]

Treatment[b]	Clean[c] (sec)	Rub[d] (sec)
Con	31.96 ± 6.42	9.04 ± 3.73
A	35.29 ± 4.17	18.86 ± 5.01
S	35.76 ± 7.71	21.60 ± 3.13
Mix	34.80 ± 8.45	17.33 ± 2.26

[a] Ten experiments of each compound were performed. Values are the means (SEM) of the absolute duration of reactions.
[b] Con = control, DMSO; A = asymmetric form, 3-hydroxy-4,5-dimethoxybenzoic acid; S = symmetric form, 4-hydroxy-3,5-dimethoxybenzoic acid; Mix = mixture of both, v/v 47:100.
[c] Time *S. comma* spent cleaning its mouthparts.
[d] Time *S. comma* spent rubbing its mouthparts on the ground.

DISCUSSION

Benzoic acid derivatives are widely distributed in nature. To our knowledge, this is the first record of both isomers 3-hydroxy-4,5-dimethoxybenzoic acid

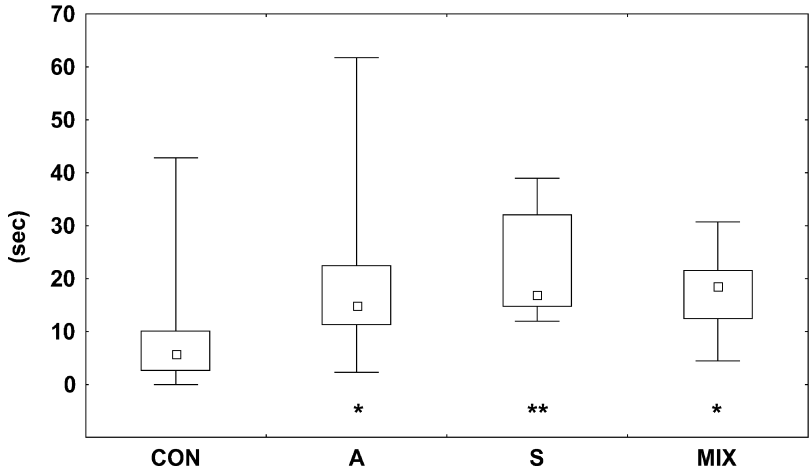


FIG. 4. Median, 25, and 75% percentiles (box) and minimum/maximum (bars) of the time *Stenus comma* spent rubbing its mouthparts on the ground within 10 min after having been topically treated with 0.5 μl of test fluid. Ten staphylinid beetle specimens were used per test fluid. In Mann-Whitney U-test compounds 3-hydroxy-4,5-dimethoxybenzoic acid (A), 4-hydroxy-3,5-dimethoxybenzoic acid (S), and a 47:100 mixture of both (MIX) vs. control (CON) were tested ($^{*}0.05 > P > 0.01$, $^{**}P < 0.01$).

and 4-hydroxy-3,5-dimethoxybenzoic acid occurring together in an organism. 3-Hydroxy-4,5-dimethoxybenzoic acid has previously only been reported from plants, e.g., black tea (Hodgson et al., 2000). 4-Hydroxy-3,5-dimethoxybenzoic acid (syringic acid) is one of the degradation products of lignin (Kovacik et al., 1969; Klinke et al., 2002). Most likely, its asymmetric isomer is also formed during lignin degradation. One could assume that the opportunistic species *C. denticulata* (Walsh and Bolger, 1990) obtains this phenolic acid from its food (litter, fungi, mushrooms, etc.) and then forms the isomeric 3-hydroxy-4,5-dimethoxybenzoic acid by enzymatic processes or by feeding on white-rot fungi that are able to produce this isomer from syringic acid (Eriksson et al., 1984). But, animals that were fed only baker's yeast also have one or both isomers, while baker's yeast in our analyses does not contain either benzoic acid derivative. Thus, it may well be that *C. denticulata* produces both isomers autogenously, especially since both compounds could not be detected in the body of the animal or in the gut. We cannot exclude the possibility that the compounds result from the presence of specific precursors in the yeast or microorganisms growing on the yeast during cultivation. However, residual phenolic acids from the field can be excluded because the collembolans were kept in culture for several generations before analysis.

The temperature dependence of the ratio of the two isomers may be explained by an enzyme in the animals that is only active at low temperatures. This enzyme may catalyze the formation of 3-hydroxy-4,5-dimethoxybenzoic acid from syringic acid or another precursor. Why this low-temperature enzymatic activity occurs, is a matter of speculation. Because of the hydrophobic properties of the isomers they could prevent ice formation on collembolan surfaces in winter, but this would not explain the need for another isomer. To clarify the function and biosynthesis of the compounds, further investigations are required, e.g., feeding of chemically labelled precursors to animals.

Insect pheromones of low volatility are often present on the surface of the cuticle (Howard and Blomquist, 1982; Nelson and Blomquist, 1995; Schulz, 2001). Production and release of pheromones or deterrents in hypogastrurid Collembola is still unclear. They do not possess pseudocells like onychiurid Collembola. Verhoef (1984) demonstrated the existence of pheromones in fecal pellets; therefore, biosynthesis is suggested to be located in or near the digestive tract of Collembola. According to Altner and Thies (1973), in *Hypogastrura socialis*, pheromone production via gland cells in the eversible sac of the antennal tip is possible. The eversible anal glands of *H. socialis* could also be a source for the release of pheromones (Leinaas, 1988). Purrington et al. (1991) report on the release of an alarm pheromone after cuticular rupture in *Hypogastrura pannosa*. This may point to the production of pheromones or deterrents in the hemolymph or, as in our case, possibly in specialized cells nearby or in the integument. If the isomers were produced in the digestive tract, we also should have found both

in the total extract. Release of pheromones via anal glands or eversible antennal sacs seems to be efficient only for highly volatile substances, but not for weakly volatile deterrents like benzoic acid derivatives.

There must exist a direct way from the interior of the body to the surface because *C. denticulata* shows reflex bleeding when it is narcotized with carbon dioxide (Bitzer, unpubl.). We suppose that the hydrophobic isomers are stored in lipid droplets near the integument and then transported to the body surface. Longitudinal sections of this collembolan species showed lots of these droplets primarily near the integument (Bitzer, unpubl.).

In bioassays with *C. denticulata*, both substances acted neither as alarm nor as aggregation pheromones. Alarm pheromones are normally highly volatile (Jutsum and Gordon, 1989). Tests with single *Hypogastrura pannosa* showed an active space for pheromones of 1 cm (Purrington et al., 1991). Similar results concerning the active space are reported from aggregation pheromones of entomobryid collembola (Verhoef et al., 1977). To exclude dose-dependent effects, we also tested higher concentrations that corresponded to 15 animals, but it had no effect on *C. denticulata*.

Tests with the collembolan predator *S. comma* showed deterrent effects of the substances. The beetles cleaned their mouthparts by rubbing them on the soil more often than in control tests. Bauer and Pfeiffer (1991) made similar observations with *S. comma* confronted with the collembolan *Podura aquatica*, but they reported nothing about the cleansing behavior. As a visual hunter, *S. comma* needs direct contact with its prey. Olfactory stimuli alone do not cause an attack. Starved *S. comma* attack living *C. denticulata*, but they immediately spit them out after contact (Bitzer, unpubl.). This underlines the existence of deterrent substances either in the integument or on the body surface of *C. denticulata*. In other insects, syringic acid also shows deterrent effects. Adams and Bernays (1978) used syringic acid in feeding experiments with *Locusta migratoria*. At high concentrations, they observed feeding reduction. The function of syringic acid as an antimicrobial compound for example in green olives is often mentioned in the literature (Tuncel and Nergiz, 1993; Aziz et al., 1998). Therefore, both isomers might also function as antimicrobial agents, especially since collembolans live in environments rich in bacteria and fungi.

Deterrents from only two collembolan species are so far known. 2-Aminophenol from *Neanura muscorum* has deterrent properties against the predatory mite *Pergamasus norvegicus* (Messer et al., 2000), while in *Tetrodontophora bielanensis* three pyridopyrazines are repellent to the carabid beetle *Nebria brevicollis* (Dettner et al., 1996).

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AVIAN EXOCRINE SECRETIONS. I. CHEMICAL CHARACTERIZATION OF THE VOLATILE FRACTION OF THE UROPYGIAL SECRETION OF THE GREEN WOODHOOPOE, *Phoeniculus purpureus*

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Abstract—Using gas chromatography–mass spectrometry in conjunction with auxiliary techniques such as solid phase microextraction and determination of double bond positions by means of dimethyl disulfide derivatization, 45 constituents of the uropygial secretion of the green woodhoopoe, *Phoeniculus purpureus*, have been identified. The majority of these constituents are long-chain branched and unbranched alkanes, and (*Z*)-alkenes such as (*Z*)-9-tricosene, and a number of unidentified wax esters. The more volatile fraction of the secretion contained short-chain fatty acids, aldehydes, aliphatic and heterocyclic aromatic amines, ketones, and dimethyl sulfides. This group of volatile compounds is responsible for the obnoxious odor of the secretion and also for its defensive action against predators.

Key Words—*Phoeniculus purpureus,* avian semiochemicals, defensive secretion, uropygial secretion.

INTRODUCTION

The uropygial gland of most birds produces a variety of hydrocarbons, lipids, waxes, fatty acids, alcohols, and other organic compounds (Jacob and Ziswiler, 1982). These have two widely recognized functions, viz. they are considered essential for the maintenance of good plumage condition, and may be used for fungicidal, bactericidal, or other hygienic purposes.

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Green woodhoopoes (also known as red-billed woodhoopoes), *Phoeniculus purpureus,* are group-territorial birds that live in groups comprising between 2 and 12 individuals (Ligon and Ligon, 1978). They are obligate cavity roosters and multiple individuals—usually all group members—will enter either a natural or bird-excavated cavity each night (Ligon and Ligon, 1978; du Plessis, 1989, 1992). Individuals enter the roost cavities shortly after sunset and exit the following morning soon after sunrise. During the period that the birds are inside the roost, they apparently are vulnerable to a range of vertebrate predators, including snakes, genets, and rats (Ligon and Ligon, 1978).

When disturbed while roosting, woodhoopoes immediately face away from the threat hence presenting their uropygial glands in the direction of the threat. Typically, a drop of brown, highly pungent secretion is then formed at the tip of the papilla to the uropygial gland, and kept in place by a few tuft-like feathers. This response pattern has led some observers to believe that the secretion serves an antipredatory role (Dallas, 1867, in Elder, 1954; Ligon and Ligon, 1978; du Plessis and Williams, 1994). The secretion has a persistent smell and frequently remains detectable for several hours on the hands of researchers who have handled them. Woodhoopoes also rank very low in terms of their taste to the human palate (Cott, 1945, 1946).

The aim of this study was to determine the chemical composition of the volatile fraction of the uropygial secretion of the green woodhoopoe, excluding the wax esters, in order to identify the volatile constituents of the secretion for evaluation of their defensive properties against predators.

METHODS AND MATERIALS

General. All Pyrex glassware used in the handling of biological material and extracts, as well as in the preparation of reference compounds, was heated to 500°C in an annealing oven to remove any traces of organic material. Dichloromethane (Merck, Residue Analysis Grade) was analyzed gas chromatographically and found to be pure enough for extraction purposes when used in small quantities. This solvent was used to clean syringes, stainless-steel needles, etc.

Secretion Collection and Sample Preparation. Uropygial gland secretion was collected either by wiping the exudates off the ring of fine bristles around the uropygial gland with pre-extracted surgical gauze squares, or by collecting the pure secretion in glass capillaries. In some instances, when the voluntary drop of secretion had been lost during capture, gentle finger pressure was applied to the base of the gland. This almost always resulted in the production of a further one to three drops of secretion. The volatile organic material was extracted from the secretion collected on gauze by extraction with dichloromethane (Reiter et al., 2003). To collect the secretion in a capillary tube, the suspended drop

of the secretion at the end of the papilla was touched with the tip of a capillary (0.5 mm i.d.), which had been scored at a point about 10 mm from its tip. Capillary forces caused the secretion to move into the capillary. The end of the capillary containing the secretion was inserted into the mouth of a small vial and broken off at the scored position. The back end of the capillary, which served as a handle during the collection process, was discarded and the vial was sealed with a PTFE-faced septum. The collected secretion normally flowed out of the capillary and covered the bottom of the vial, and was sampled with solid phase microextraction (SPME) or was extracted with 100 μl of dichloromethane. Extracts could mostly be used without evaporation of the solvent. When more concentrated solutions were required, the solvent was removed with a slow stream of purified (activated charcoal) nitrogen.

Analytical Methods. Gas chromatographic (GC) analyses were carried out with Carlo Erba 4200 and 5300 gas chromatographs equipped with flame-ionization detectors and Grob split/splitless injectors. Glass capillary columns were manufactured by the Laboratory for Ecological Chemistry and were coated with 0.25 μm of the apolar stationary phase PS-089 (DB-5 equivalent) or with 0.375 μm of OV-1701. Hydrogen was used as carrier gas at a linear velocity of 50.0 cm/sec at 40°C. The flame-ionization detector was operated at 280°C, and the injector was normally used at 220°C. For qualitative work, samples of secretion extracts were injected in the split mode, the volatiles entering the column were cold trapped at ca. 30°C, and subsequently analyzed using a temperature program of 4°C/min from 40 to 280°C (hold). Quantitative analyses were done with on-column introduction of aliquots of the extracts and the external standards, butyl ethyl ether and 1-icosene.

A solventless sample introduction probe (Burger et al., 1990a) was used for sample introduction in GC and GC–MS analyses, and headspace analyses were done by SPME (Zhang and Pawliszyn, 1993) using a 100-μm polydimethylsiloxane fiber. Headspace sampling times from 7 hr at 22°C to 72 hr at 40°C were employed.

Low-resolution electron impact mass spectra (EI-MS) were obtained at 70 eV on a Carlo Erba QMD 1000 GC–MS instrument using the above columns and conditions. The injector temperature was set at 220°C, the interface temperature at 250°C, and the ion source temperature at 180°C. A scan rate of 0.9 sec/scan with an interval of 0.1 sec between scans was employed. Helium was used as carrier gas at a linear velocity of 28.6 cm/sec at 40°C.

^{1}H and ^{13}C NMR spectra of the total secretion and synthesized reference compounds were obtained on a Varian VXR-300 spectrometer at 299.9 MHz and 75.42 MHz, respectively.

Determination of Double Bond Positions of Constituents. Dimethyl disulfide (DMDS) derivatives of the unsaturated constituents of the secretion were prepared according to Buser et al. (1983). An aliquot (10 μl) of a dichloromethane extract

of the uropygial secretion was concentrated in a 1-ml Reacti-Vial using a slow stream of purified nitrogen. The residual material was dissolved in hexane (50 μl), and treated with 5 μl of iodine solution (60 mg/ml in diethyl ether) and dimethyl disulfide (50 μl). The Reacti-Vial was sealed using a PTFE-faced rubber septum, and the reaction mixture was warmed for 15 hr at 40°C in the oven of a gas chromatograph, after which the reaction was quenched with an aqueous solution of sodium thiosulfate (5%). The organic and aqueous layers were separated by centrifuging for a few min at 2000 rpm, and the organic layer was transferred to a clean Reacti-Vial. The solution was concentrated to ca. 5 μl for GC–MS analysis.

Reference Compounds. Compounds required for comparison with constituents of the uropygial secretion that were not commercially available were synthesized. (*Z*)-7-Henicosene was synthesized from 1-octyne and 1-bromotridecane (Ziegenbein, 1969), followed by catalytic hydrogenation of the resulting alkyne with Lindlar catalyst to yield the corresponding olefin. This procedure was also used for the synthesis of all the other alkenes used for retention time comparison with the natural compounds.

RESULTS AND DISCUSSION

Employing collection of the secretion in a capillary, about 5 mg of the secretion could be collected from birds that had not lost any of the secretion during capture or handling. The secretion consisted largely of water and evaporation of the volatile material left only a foul smelling yellow stain on a glass surface. A typical total ion chromatogram obtained by GC–MS analysis of an extract of the secretion of an adult male is shown in Figure 1. Tentative identifications of the volatile organic constituents of the secretion were based on comparison of their mass spectra with those in the available NBS and Wiley mass spectra libraries. The compounds identified are listed in Table 1 together with information on the analytical techniques employed in their identification and some quantitative data on the major constituents present in the secretion in quantities higher than 0.1 μg/animal.

Because the more volatile short-chain constituents of the secretion have characteristic mass spectra, their identification was simple. However, a large proportion of the constituents of the secretion were relatively large saturated and monounsaturated hydrocarbons. Of these, the unbranched isomers were identified by their distinctive MS fragmentation patterns and by co-elution with synthetic reference compounds. In the case of the methyl-branched components, the characteristic ions formed by fragmentation at branch points provided positive identification.

The positions of the double bonds in the monounsaturated hydrocarbons were determined by GC–MS analysis of an extract of the secretion that had been subjected to dimethyl disulfide derivatization (Buser et al., 1983). The

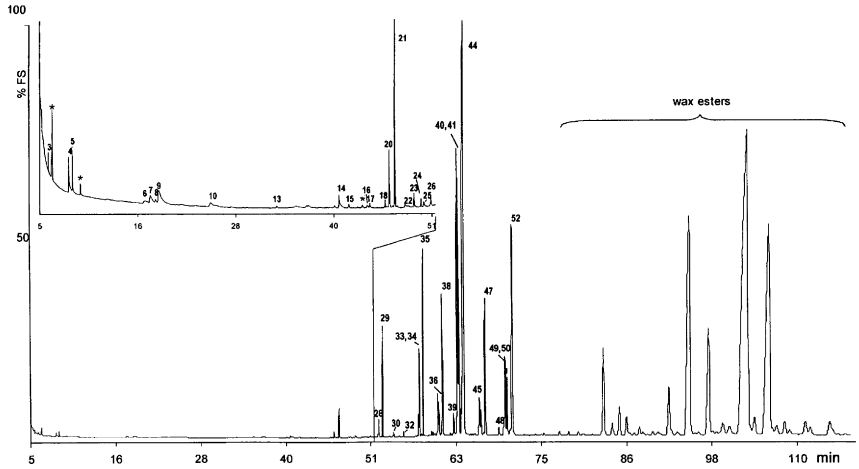

FIG. 1. Total ion chromatogram (TIC) of an extract of the uropygial secretion of the green woodhoopoe *Phoeniculus purpureus*. Gas chromatographic conditions: 40 m × 0.3 mm ID glass column coated with PS-089 at a film thickness of 0.25 μm and programed at 2°C from 40° to 250°C (hold); injector temperature 220°C.

characteristic fragments of the resulting bis(methylthioethers) are derived by the cleavage through the position of the (former) double bond of the alkene, the ion of higher mass having a higher intensity than the lower mass ion in all cases (Francis and Veland, 1981). Another prominent diagnostic ion in the spectra of these derivatives is the (M-47) ion, formed by loss of a methylthio radical from the molecular ion.

The configurations of the double bonds of the major unsaturated constituents of the secretion were established by NMR spectrometry. It is known that the allylic methylene carbon atoms resonate at δ ca. 27 and at δ ca. 33 in the ^{13}C-NMR spectra of long-chain unbranched *Z*- and E-olefins, respectively (Wenkert et al., 1976; Breitmaier et al., 1979). The presence of a resonance at δ 27.9 in the ^{13}C-NMR spectrum of the secretion and the absence of a signal at about δ 33 was therefore construed as evidence that the major unsaturated constituents of the secretion possessed *Z* configuration. This was confirmed by the typical vicinal coupling constants of (*Z*)-olefins of 11.2 Hz (Hesse et al., 1987) observed in the ^{1}H-NMR-spectrum of the secretion. The (*E*)- and (*Z*)-isomers of the olefins present in the secretion were separable on the capillary columns used in the study and the configuration of the double bonds in these alkenes was confirmed by GC co-elution of a number of synthetic standards with some of the olefins in the secretion.

TABLE 1. COMPOUNDS IDENTIFIED IN THE UROPYGIAL SECRETION OF GREEN WOODHOOPOE

No. in Fig. 1	Compound	Identification	μg/animal[a]
1	Trimethylamine	a,b	c
2	Propanoic acid	a,b	c
3	3-Methylbutanal	a,b	0.9
4	3-Methylbutan-1-ol	a,b	3.0
5	Dimethyl disulfide	a,b	3.7
6	4-Methylpentanoic acid	a,b	0.4
7	Benzaldehyde	a,b	2.0
8	Dimethyl trisulfide	a,b	0.1
9	Phenol	a,b	1.4
10	2-Phenylethanol	a,b	
11	2-Phenylethyl acetate	a,b	
12	Indole	a,b	
13	Tridecane	a,b	
14	Pentadecane	a,b	
15	Unidentified		
16	Hexadecane	a,b	
17	Unidentified		
18	2-Methylhexadecane	a	0.5
19	(*Z*)-Heptadec-7-ene	a,e	
20	(*Z*)-Heptadec-9-ene	a,e	3.4
21	Heptadecane	a,b	12.5
22	13-Methyltetradecan-1-ol	a,d	0.5
23	3-Methylheptadecane	a	0.9
24	Octadecane	a,b	0.7
25	Hexadecanal	a,b	0.5
26	14-Methylpentadecan-1-ol	a,b	2.1
27	(*Z*)-Nonadec-7-ene	a,e	2.4
28	(*Z*)-Nonadec-9-ene	a,e	9.4
29	Nonadecane	a,b	64.7
30	5-Methylnonadecane	a	6.5
31	(*Z*)-Icos-9-ene	a,e	1.4
32	Icosane	a,b	3.7
33	(*Z*)-Henicos-7-ene	a,b,e	16.0
34	(*Z*)-Henicos-9-ene	a,b,e	65.6
35	Henicosane	a,b	204
36	(*Z*)-Docos-7-ene	a,e	33.8
37	(*Z*)-Docos-9-ene	a,e	26.1
38	Docosane	a,b	143.8
39	Unidentified $C_{23}H_{44}$	a	22.4
40	Unidentified $C_{23}H_{44}$	a	15.2
41	(*Z*)-Tricos-7-ene	a,e	422
42	(*Z*)-Tricos-9-ene	a,b,e	306
43	Unidentified tricosene	a	12.6
44	Tricosane	a,b	2570
45	(*Z*)-Tetracos-7-ene	a,e	86.7

TABLE 1. CONTINUED

No. in Fig. 1	Compound	Identification	μg/animal[a]
46	(*Z*)-Tetracos-9-ene	a,e	1.1
47	Tetracosane	a,b	211
48	Unidentified $C_{25}H_{48}$	a	13.1
49	Unidentified $C_{25}H_{48}$	a	20.1
50	(*Z*)-Pentacos-7-ene	a,e	120
51	(*Z*)-Pentacos-9-ene	a,e	85.9
52	Pentacosane	a,b	575

[a] Quantities lower than 0.1 μg are not given. a, GC–MS analysis; b, retention time comparison with standard compounds (purchased or synthesized); c, observed in SPME analysis only; d, GC–MS analysis, retention time increment comparison; e, DMDS-derivatization

GC–MS analyses of extracts of the secretion gave total ion chromatograms showing many constituents with retention times between 75 and 120 min (Figure 1). These compounds were found to be wax esters, which are likely to waterproof the feathers during preening. Most birds regularly rub their bills over the uropygial gland while grooming their feathers (Jacob and Ziswiler, 1982). It was relatively easy to derive the carbon numbers of the fatty acid and alcohol moieties of these esters from their mass spectra, which contained, *inter alia*, weak molecular ions. However, reference compounds synthesized from unbranched starting materials eluted with longer retention times than the natural compounds. The natural substances therefore have to be branched isomers, which have not yet been identified. However, it is possible that branching of the type present in some of the volatile constituents of the secretion could also be present in these wax esters. Because of their low volatility, these compounds did not appear in total ion traces of SPME extracts from the headspace of the secretion, even when sampling was continued for up to 72 hr. The more volatile compounds, on the other hand, were present in higher relative amounts when headspace samples were analyzed.

In general, only minor quantitative differences were observed between the secretions collected from different males and females. No consistent variation could be found between ranking and sex of the birds. However, the secretion of one adult female was found to have a large preponderance of the various isomers of the fatty acids up to pentanoic acid. It is, therefore, possible that other, at this stage unknown, factors could have an influence on the quantitative composition of the secretion. The short-chain fatty acids, aldehydes, trimethylamine, indole, and dimethyl disulfide are responsible for the pungently unpleasant smell of the secretion.

The occurrence of several volatile compounds, a wide variety of hydrocarbons, in particular monounsaturated olefins such as (*Z*)-9-tricosene and

(*Z*)-9-henicosene, as well as heavy wax esters, gives the impression that the uropygial secretion of the green woodhoopoe may function as more than a water proofing component for feathers. In insect and mammalian exocrine secretions, the active principles are often produced as mixtures with proteinaceous or nonvolatile lipids that act as controlled release materials (Burger et al., 1990b, 1997). For example, nonvolatile lipids have been found in the uropygial secretion of grebes (Jacob, 1978). It is possible that the lipid constituents of the uropygial secretion could fulfill a similar function in the green woodhoopoe. However, it is doubtful that this is the primary function of the lipid constituents of the woodhoopoe's secretion, because this secretion contains too little material to be of much use as a controlled release carrier. The secretions of other animals using this dissemination mechanism contain a much larger proportion of either proteinaceous or lipid material.

The role of the uropygial material as a defensive secretion was recently confirmed in field tests. A formulation of a selection of the volatile representatives of each of the different compound classes in the secretion was found to be effective against feline predators and lizards (du Plessis et al., unpublished results). The full synthetic secretion was also tested against pathogenic and parasitic bacterial species identified from nest sites of birds and was found to be effective against several of these species, including *Bacillus licheniformis*, which degrades feathers (Burtt, 1999). This bacterium could be potentially lethal due to loss of insulation and impairment of the flight ability of birds. *B. licheniformis* showed a higher susceptibility to the synthetic secretion than any of the other pathogenic bacteria tested. The uropygial secretion of the woodhoopoe, therefore, not only protects and waterproofs feathers but may also inhibit the growth of feather degrading bacteria.

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FIELD-TESTING OF METHYL SALICYLATE FOR RECRUITMENT AND RETENTION OF BENEFICIAL INSECTS IN GRAPES AND HOPS

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Abstract—Evidence for recruitment and retention of beneficial insects in grapes and hops using controlled-release dispensers of methyl salicylate (MeSA), a component of herbivore-induced volatile blends, is presented. In a replicated experiment conducted in a juice grape vineyard, sticky cards in blocks baited with MeSA captured significantly greater numbers of five species of predatory insects (*Chrysopa nigricornis, Hemerobius* sp., *Deraeocoris brevis, Stethorus punctum picipes, Orius tristicolor*) than unbaited blocks. Four insect families (Syrphidae, Braconidae, Empididae, Sarcophagidae) were also significantly more abundant in the MeSA-baited blocks, as indicated by sticky card captures. Canopy shake samples and sticky card monitoring conducted in a MeSA-baited, unsprayed hop yard indicated development and maintenance of a beneficial arthropod population that was nearly four times greater than that present in an unbaited reference yard. Four times as many *S. punctum picipes* and six times as many *O. tristicolor* were sampled in the MeSA yard. Similar contrasts in abundance of these predators and others were apparent when compared with levels recorded in the yard in previous years. The large population of predatory insects in the MeSA-baited hop yard was associated with a dramatic reduction in spider mite numbers, the major arthropod pest of hops, in late June, and subeconomic populations were maintained for the rest of the season. The evidence presented here is highly suggestive that the use of controlled-release MeSA in a crop could increase recruitment and residency of populations of certain beneficial insects. This strategy may have the potential to enhance the efficacy and reliability of conservation biological control in crop pest management.

Key Words—Methyl salicylate, herbivore-induced plant volatiles, hops, grapes, beneficial insects, conservation biological control.

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INTRODUCTION

The phenolic compound, methyl salicylate (MeSA), has been identified in the herbivore-induced volatile blends from at least 13 crop plant species including bean, cowpea, hops, tomato, cucumber, cabbage, pear, bird-cherry, sweet pepper, grape, and potato (see James, 2003a, for summary; Van Den Boom et al., 2004). Tobacco plants inoculated with tobacco mosaic virus also produced MeSA (Shulaev et al., 1997). Wild tobacco, *Nicotiana attenuata* Torr., plants attacked by hornworm larvae showed elevated levels of MeSA emission (Kessler and Baldwin, 2001), as did two other noncrop plants, *Datura stramonium* L. and *Robinia pseudo-acacia* L. when damaged by the spider mite, *Tetranychus urticae* Koch (Van Den Bloom et al., 2004).

Laboratory studies have demonstrated MeSA to be attractive to the predatory mite, *Phytoseiulus persimilis* Athias-Henriot (Phytoseiidae) (Dicke and Sabelis, 1988; Dicke et al., 1990; Ozawa et al., 2000a) and the predatory bug, *Anthocoris nemoralis* (Fabricius) (Anthocoridae) (Drukker et al., 2000). In contrast, MeSA was shown to repel aphid pests in the laboratory (Hardie et al., 1994; Petterson et al., 1994) and in the field (Losel et al., 1996; Bernasconi et al., 1998; Glinwood and Petterson, 2000; Ninkovic et al., 2003). Shulaev et al. (1997) suggested that MeSA, (the volatile form of salicylic acid, an endogenous plant signal implicated in eliciting plant resistance (Ryals et al., 1996)), is a key compound in the induced resistance of tobacco plants to pathogens. Evidence of a plant–plant signaling role for MeSA was also provided by Ozawa et al. (2000b) who demonstrated that lima bean leaves exposed to the compound produced a blend of volatiles similar to that caused by spider mite feeding.

Field evidence for attraction of natural enemies to herbivore-induced plant volatiles is limited. Drukker et al. (1995) found increased numbers of anthocorid predators near cages with *Psylla*-infested pear trees. Similarly, Shimoda et al. (1997) recorded more predatory thrips on sticky cards near spider mite-infested bean plants than on traps near uninfested plants. Bernasconi et al. (2001) trapped more natural enemies near plants damaged and treated with caterpillar regurgitant, than near undamaged, untreated plants. Direct evidence for the potential of synthetic plant volatiles as field attractants for beneficial insects was obtained by James (2003a,b) who demonstrated attraction of a number of species and families to MeSA and (*Z*)-3-hexenyl acetate. Insects attracted to MeSA included *Chrysopa nigricornis* Burmeister (Chrysopidae), *Geocoris pallens* Stal. (Geocoridae), *Stethorus punctum picipes* (Casey) (Coccinellidae), and species of Syrphidae. These beneficial insects are important components of conservation biological control programs currently being developed for vineyards and hop yards in Washington State (James et al., 2003). The possibility that MeSA dispensed from controlled-release dispensers could have potential in pest management by increasing populations of beneficial insects in crops

like hops and grapes, thereby improving biological control, is the subject of this paper.

METHODS AND MATERIALS

Vineyard Experiment. This was conducted in an unsprayed 10 ha juice grape (var. Concord) vineyard located near Prosser in south-central Washington State during spring to autumn (May–September) 2003. Controlled-release plastic sachets containing 5 g of MeSA (98%) were prepared and supplied by Chem-Tica Internacionale (Costa Rica). Under laboratory conditions the sachets released 30 mg of MeSA per day, but in the field under summer conditions in eastern Washington (daily maxima 28–40°C), release of up to 60 mg/day is likely (Allan Oehlschlager, pers. Comm.). The sachets were deployed in three of six 8 × 30 m blocks randomly distributed in the vineyard. Each block contained 65 grapevines (5 rows of 13 vines), and blocks were separated by a minimum of 100-m distance. In the MeSA blocks, 55 sachets were singly stapled on alternate supporting posts, providing an approximate density equivalent to 2297/ha. Three blocks containing no MeSA dispensers served as unbaited controls. Populations of beneficial insects were monitored using three yellow sticky cards (23 × 18 cm, Trece Incorporated, Salinas, CA) per block, equally spaced (8 m apart) along the center row. Cards were collected and replaced weekly with insects identified and counted in the laboratory under a stereomicroscope. Identifications were made to species where possible but a number of groups were only identified to family.

Hop Yard Experiment. This was conducted during May–September 2003 in a 0.81 ha commercial hop yard that was not exposed to insecticides, although some fungicides were applied. Methyl salicylate dispensers (as described above) were stapled to supporting poles (2/pole) on April 28 throughout the yard at a density equivalent to 448/ha. The principal arthropod pests of hops in Washington, twospotted spider mite, *Tetranychus urticae* Koch (Tetranychidae) and the hop aphid, *Phorodon humuli* Schrank (Aphididae), and all beneficial arthropods were monitored weekly by sampling leaves, canopy "shaking," and retrieving/replacing five yellow sticky cards stapled to poles. Thirty leaves were collected randomly at 1.5 m above ground level from throughout the yard and placed in a plastic bag for transport to the laboratory in a cooler. Leaves were examined under a stereomicroscope, and numbers of mites and aphids were recorded. Canopy "shake sampling" was conducted by vigorously shaking a hop vine over a 1 m^2 collection funnel for 3 sec. Arthropods were brushed/tapped into a collecting jar at the base of the funnel containing ethyl alcohol. Arthropods in the jars were sorted, identified, and counted under a stereomicroscope in the laboratory. Nine vines (three vines in each of three randomly selected locations) were shaken each week (from June 23 onwards, when vines had enough canopy) to provide three

samples. Sticky cards were positioned in a line in the center of the yard and separated by at least 10 m. After collection, they were examined in the laboratory with all beneficial insects identified and counted. Arthropod sampling data were also collected from this yard (using the same techniques) for earlier years (leaves: 1999–2002, shake samples: 2001/2002, sticky traps: 2002). The only insecticide or miticide used in this yard in 2001 and 2002 was bifenazate, a compound that is relatively safe to most beneficial insects in hops (James and Coyle, 2001; James, 2002). No nearby insecticide-free hop yards were available in 2003 to provide a strict "control" for the MeSA-baited yard. However, one hop yard located about 5 miles away used only single applications of a miticide (bifenazate) and an aphicide (pymetrozine). Pymetrozine is also safe to beneficial insects (James, 2002). We therefore used this site as a "reference" biological control yard for comparison to the MeSA-baited yard by conducting weekly leaf and canopy shake sampling during the same period as for the MeSA-baited yard. Sampling data from both experiments were log $(x + 1)$ transformed to equalize variance and analyzed using either the Mann–Whitney Rank–Sum Test, or the Kruskal–Wallis ANOVA on ranks.

RESULTS

Vineyard Experiment. Arthropod herbivory was minor in the vineyard apart from some leaf damage caused by leafhoppers (*Erythroneura* spp.) late in the season. Methyl salicylate could be smelled in the baited blocks during May–July. In August–September, odor was only apparent when dispensers were inspected closely. Analyses conducted for the entire period revealed significantly greater numbers of three species of predatory insects (*Chrysopa nigricornis, Hemerobius* sp. (Hemerobiidae), *Deraeocoris brevis* (Uhler) (Miridae)) were trapped in the MeSA-baited blocks than in the unbaited blocks (Table 1, Figure 1). *Geocoris pallens, Stethorus punctum picipes*, and *Orius tristicolor* (White) (Anthocoridae) were significantly more numerous in the MeSA-baited blocks during July–September but not during May–June (Table 1, Figure 2). Most of these species showed a trend of increasing abundance in the MeSA (but not the control) blocks as the season progressed. Four insect families, Syrphidae (hover flies), Braconidae (wasps), Empididae (dance flies), and Sarcophagidae (flesh flies) were also significantly more abundant on sticky cards in MeSA-baited blocks (Table 1). Significantly greater numbers of "parasitic wasps" (categorized as small (<2 mm in length) black or yellow wasps) were also trapped in the MeSA-baited blocks (Table 1). Other insects trapped that did not show significantly greater abundance in MeSA-baited blocks included Ichneumonidae (wasps), Dolichopodidae (long-legged flies), Chloropidae (Frit flies), Coccinellidae (ladybeetles excluding *S. punctum picipes*), *Lygus hesperus* Knight (Miridae), *Leptothrips mali* (Fitch)

TABLE 1. SEASON (MAY–SEPTEMBER; UNLESS OTHERWISE INDICATED) (MEAN (±SE) INCIDENCE OF BENEFICIAL INSECTS TRAPPED ON STICKY CARDS LOCATED IN GRAPE BLOCKS BAITED WITH CONTROLLED-RELEASE METHYL SALICYLATE DISPENSERS, AND IN UNBAITED BLOCKS

Beneficial insect	Methyl salicylate	Unbaited	*P*
Chrysopa nigricornis	0.38 (0.08)[a]	0.17 (0.04)	0.05
Hemerobius sp.	0.23 (0.06)[a]	0.16 (0.009)	0.03
Stethorus punctum picipes			
May–September	0.34 (0.10)	0.11 (0.02)	0.10
July–September	0.43 (0.08)[a]	0.11 (0.03)	0.03
May–June	0.22 (0.13)	0.10 (0.03)	0.90
Orius tristicolor			
May–September	0.45 (0.07)	0.21 (0.03)	0.12
July–September	0.66 (0.11)[a]	0.18 (0.04)	0.004
May–June	0.16 (0.05)	0.25 (0.06)	0.33
Geocoris pallens			
May–September	0.13 (0.03)	0.09 (0.02)	0.47
July–September	0.12 (0.01)[a]	0.02 (0.02)	0.05
May–June	0.17 (0.05)	0.22 (0.05)	0.69
Deraeocoris brevis	0.19 (0.06)[a]	0.07 (0.04)	0.05
Syrphidae	0.46 (0.08)[a]	0.24 (0.05)	0.03
Braconidae	1.57 (0.14)[a]	0.69 (0.07)	0.005
Sarcophagidae	11.51 (1.50)[a]	3.10 (0.49)	<0.001
Empididae	13.78 (1.29)[a]	4.77 (0.51)	0.002
"Parasitic wasps"	34.79 (4.60)[a]	24.39 (3.14)	<0.001
Lygus hesperus	0.19 (0.04)	0.39 (0.09)	0.70
Leptothrips mali	1.97 (0.34)	1.67 (0.33)	0.10
Frankliniella occidentalis	520.90 (36.0)	505.90 (55.0)	0.10
Anagrus spp.	21.00 (2.10)	32.80 (2.90)	0.12
Dolichopodidae	0.72 (0.11)	0.45 (0.06)	0.12
Ichneumonidae	0.65 (0.08)	0.61 (0.10)	0.66
Coccinellidae (excluding *S. punctum picipes*)	0.13 (0.03)	0.11 (0.03)	0.82
Chloropidae	1.17 (0.19)	1.32 (0.21)	0.39

[a] Significantly greater than corresponding value for unbaited blocks (Mann–Whitney Rank-Sum Test).

Phlaeothripidae), *Frankliniella occidentalis* Pergande (Thripidae), and *Anagrus* spp. (Mymaridae) (Table 1).

Hop Yard Experiment. Total abundance of predatory insect species in canopy shake samples was significantly greater in the MeSA-baited yard than in the unbaited reference yard (Table 2, Figure 3). Two important spider mite predators, *S. punctum picipes* and *O. tristicolor*, were also significantly more abundant in canopy shake samples from the MeSA-baited yard (Table 2, Figure 4). Comparisons of abundance of both species in this yard between 2003, 2002, and 2001, indicated significantly greater numbers in 2003 (Table 3, Figure 5). Lacewing

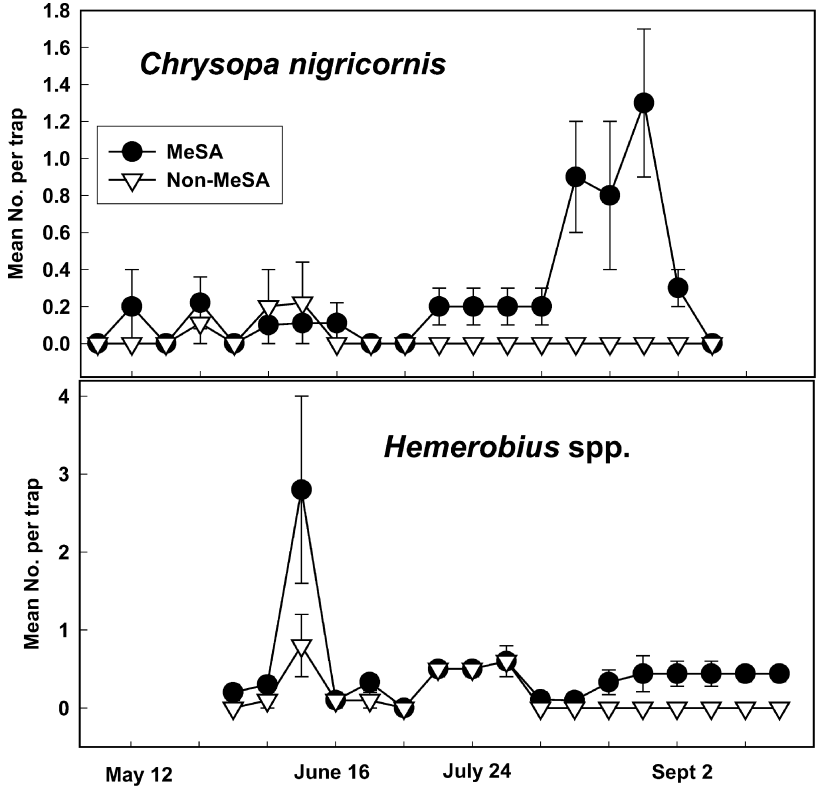

FIG. 1. Weekly mean (±SE) abundance of lacewings (*Chysopa nigricornis, Hemerobius* spp.) on sticky cards in methyl salicylate-baited and unbaited blocks in a juice grape vineyard during May–September 2003.

(*C. nigricornis* and *Hemerobius* spp.) abundance showed a similar difference between the years (Table 3, Figure 6). Sticky trap data for *S. punctum picipes* and *O. tristicolor* in 2002 and 2003 also indicated significantly greater abundance of these predators in 2003 (Table 3, Figure 7).

Spider mites in the MeSA-baited yard increased during early June and reached a mean of 67 mites/leaf on June 17. Within a week, numbers fell to 5/leaf and remained at low levels for the rest of the season (Figure 8). In the four earlier seasons, mite numbers exceeded 10 mites/leaf (economic threshold used by hop growers) for prolonged periods and miticides were applied (Figure 8). Aphid numbers in the yard showed an identical trend to spider mites. Numbers peaked at 55/leaf on June 9 and fell to 7/leaf by the end of the month and stayed at subeconomic levels for the rest of the season.

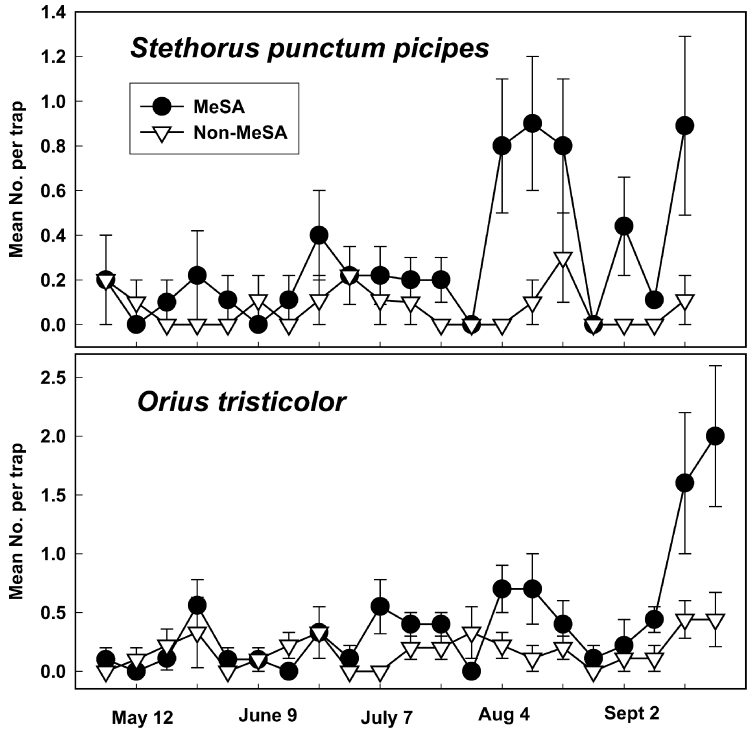

FIG. 2. Weekly mean (±SE) abundance of *Stethorus punctum picipes* and *Orius tristicolor* on sticky cards in methyl salicylate-baited and unbaited blocks in a juice grape vineyard during May–September 2003.

DISCUSSION

The data presented here are the first to show that use of a synthetic herbivore-induced plant volatile (methyl salicylate) in a crop dispensed via controlled-release

TABLE 2. SEASON (MAY–SEPTEMBER) MEAN (±SE) INCIDENCE OF BENEFICIAL INSECTS IN CANOPY SHAKE SAMPLES COLLECTED IN A METHYL SALICYLATE-BAITED HOP YARD AND AN UNBAITED HOP YARD

Beneficial insect	Methyl salicylate	Unbaited	*P*
All predators	56.80 (7.5)[a]	14.5 (1.9)	<0.001
Stethorus punctum picipes	4.03 (1.0)[a]	0.07 (0.05)	<0.001
Orius tristicolor	19.17 (2.69)[a]	2.67 (0.77)	<0.001

[a] Significantly greater than corresponding value for unbaited yard (Mann–Whitney Rank-Sum Test).

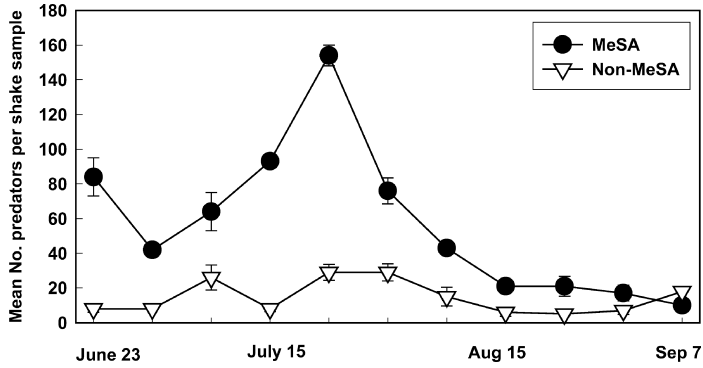

FIG. 3. Weekly mean (±SE) abundance of all predatory insects species collected in canopy shake samples from a methyl salicylate-baited hop yard and an unbaited yard during June–September 2003.

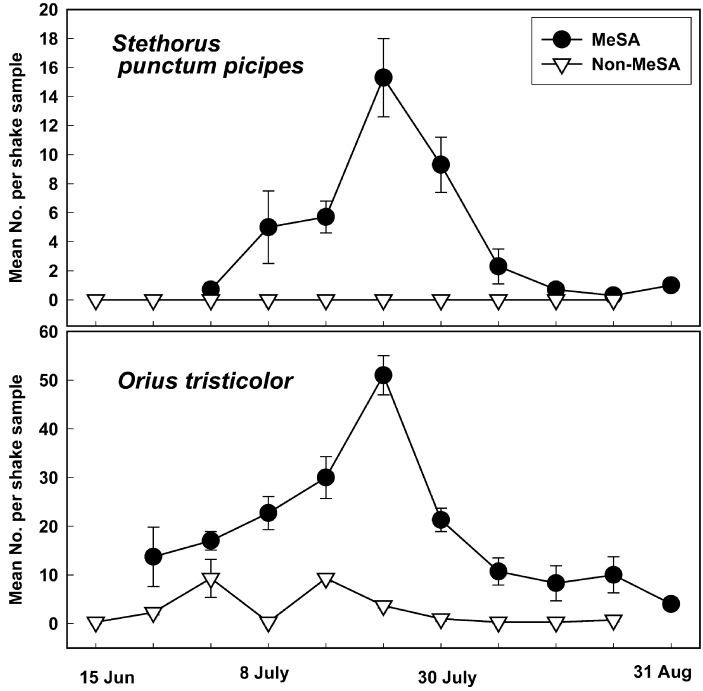

FIG. 4. Weekly mean (±SE) abundance of *Stethorus punctum picipes* and *Orius tristicolor* collected in canopy shake samples from a methyl salicylate-baited hop yard and an unbaited yard during June–September 2003.

TABLE 3. SEASON (MAY–SEPTEMBER) MEAN (±SE) INCIDENCE OF BENEFICIAL INSECTS IN CANOPY SHAKE SAMPLES AND ON STICKY CARDS IN A HOP YARD BAITED WITH METHYL SALICYLATE IN 2003 AND IN THE SAME YARD IN 2001 AND 2002 (UNBAITED)

	Canopy shake			
Beneficial insect	2001	2002	2003	*P*
Chrysopa nigricornis & *Hemerobius* sp.	0.09 (0.09)	2.11 (0.56)	4.74 (1.10)[a]	0.037
Stethorus punctum picipes	0.20 (0.10)	1.00 (0.90)	6.10 (1.30)[a]	0.016
Orius tristicolor	1.28 (0.40)	3.10 (1.00)	19.20 (2.70)[a]	<0.001
		Sticky cards		
		2002	2003	*P*
Chrysopa nigricornis & *Hemerobius* sp.		0.14 (0.06)	0.94 (0.34)[b]	0.022
Stethorus punctum picipes		0.42 (0.12)	14.80 (3.10)[b]	<0.001
Orius tristicolor		1.33 (0.36)	4.30 (0.99)[b]	0.011

[a] Significantly greater than corresponding values for other years (Kruskal–Wallis ANOVA).
[b] Significantly greater than corresponding value for 2002 (Mann–Whitney Rank-Sum Test).

sachets may increase recruitment and residency of some beneficial insects. The vineyard experiment, showed positive responses by certain insect species and families to MeSA-baited areas. The hop yard experiment, while nonreplicated, provided evidence, for enhancement of populations of some predatory insect species. Further, our results suggested that biological control of spider mites was improved by MeSA in the hop experiment.

Some of the insects that showed a significant response to MeSA were shown previously to respond to MeSA-baited sticky cards (e.g., *C. nigricornis*, *G. pallens, S punctum picipes*, and Syrphidae) (James, 2003a,b). Brown lacewings (*Hemerobius* spp.) were shown to respond to MeSA for the first time, and the anthocorid bug, *O. tristicolor*, responded to MeSA in both experiments. In James (2003b), *O. tristicolor* was reported as attracted to sticky cards baited with (*Z*)-3-hexenyl acetate but not to MeSA. However, a significant response to MeSA was noted in one month (July) of that study (James, 2003a). In the current study, MeSA also attracted wasps in the family Braconidae and flies in two families (Empididae, Sarcophagidae). Braconids and empidids are beneficial insects with the former being important parasitoids of many herbivores including caterpillars, flies, and true bugs. Empidids are predatory as adults (mainly on other flies) and larvae but their biology is generally little known. Most sarcophagids (flesh flies) feed as larvae on animal flesh, although some species are parasitoids of herbivores like grasshoppers. Attraction of these families to MeSA has not previously been recorded.

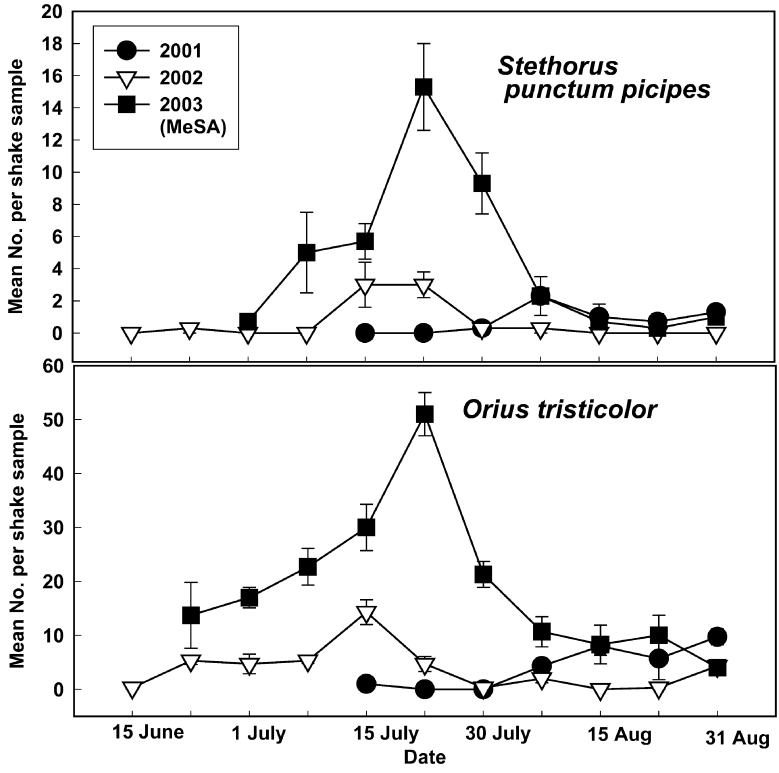

FIG. 5. Weekly mean (±SE) abundance of *Stethorus punctum picipes* and *Orius tristicolor* collected in canopy shake samples from a methyl salicylate-baited hop yard in 2003 and from the same yard in 2001 and 2002 when methyl salicylate was not used.

The response to MeSA in the vineyard experiment was greater for some species (e.g., *S. punctum picipes, O. tristicolor, C. nigricornis, D. brevis, G. pallens*) during July–September, compared to May–June. This may simply have been a reflection of low abundance of these species during spring, or it may have been a response to changing MeSA atmospheric concentrations due to sachet depletion and/or increased volatilization/dispersal caused by higher summer temperatures. The lack of detectable odor in the baited blocks during July–September, compared to the strong odor present in May–June was obvious. The density of dispenser deployment was approximately five times greater in the vineyard than in the hop yard experiment. Atmospheric concentrations of MeSA may, therefore, have been excessive and perhaps inhibitory to attraction of predators in the vineyard, during the first two months. Clearly, the question of optimal atmospheric concentrations for predator attraction is critical and needs study.

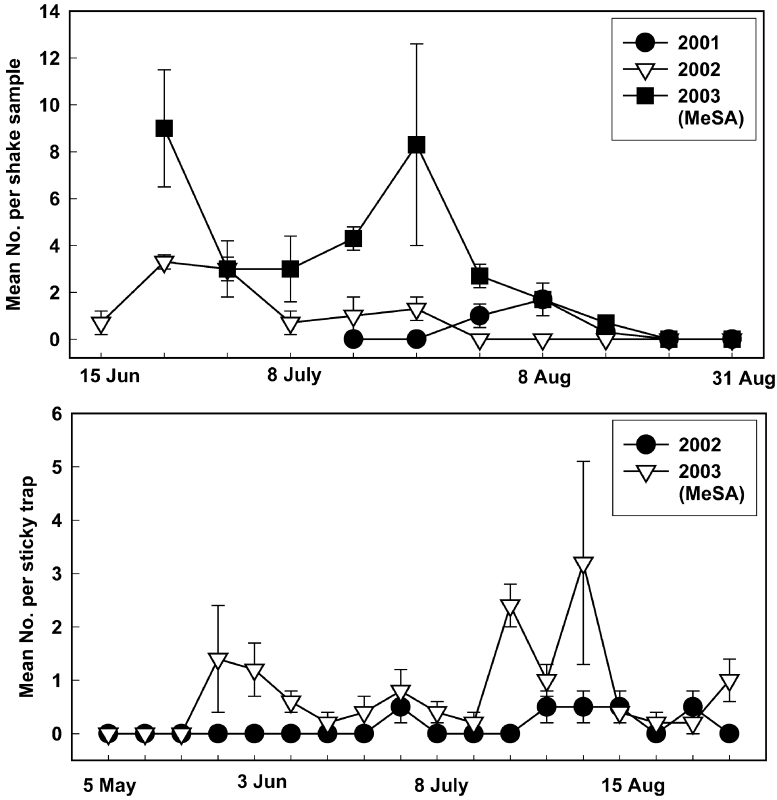

FIG. 6. Weekly mean (±SE) abundance of lacewings (*Chrysopa nigricornis* and *Hemerobius* sp.) in canopy shake samples (upper graph) and on sticky cards (lower graph) in a methyl salicylate-baited hop yard in 2003 and in the same yard in 2001 and 2002 when methyl salicylate was not used.

The abundance of predatory insects collected in the canopy shake samples in the MeSA-baited hop yard was nearly four times greater than their abundance in the reference yard. Similarly, four times as many *S. punctum picipes* and six times as many *O. tristicolor* were collected in the MeSA-baited yard. Similar contrasts in the abundance of these two predators, as well as of lacewings, were also evident when numbers from previous years in the MeSA-baited yard were compared with 2003. The high levels of beneficial insect abundance in the yard in 2003 appeared to cause the suppression of spider mites observed from late June onwards. The decline in spider mite numbers during the third week of June from 69 to 5 mites/leaf was unprecedented. Biological control of spider mites in Washington hops from this high level characteristically takes two or three weeks

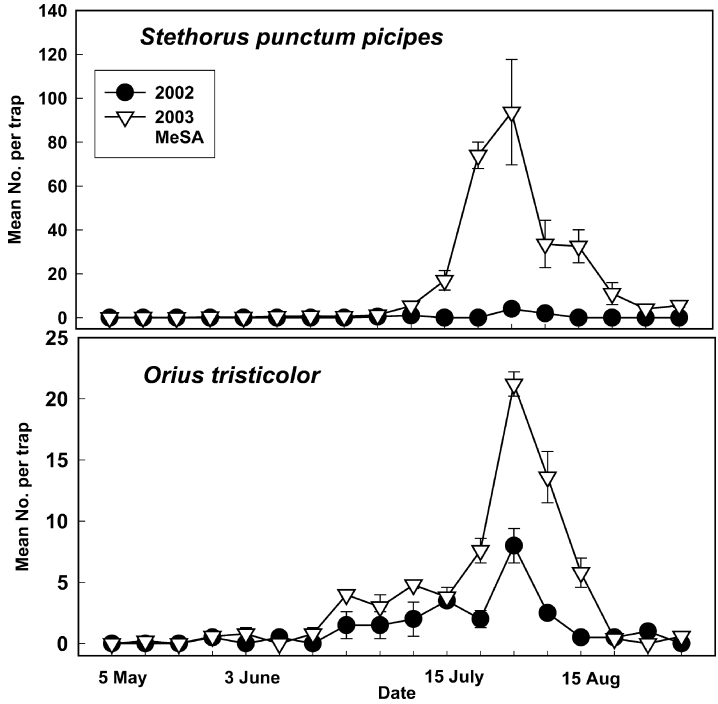

FIG. 7. Weekly mean (±SE) abundance of *Stethorus punctum picipes* and *Orius tristicolor* on sticky cards in a methyl salicylate-baited hop yard in 2003 and in the same yard in 2002 when methyl salicylate was not used.

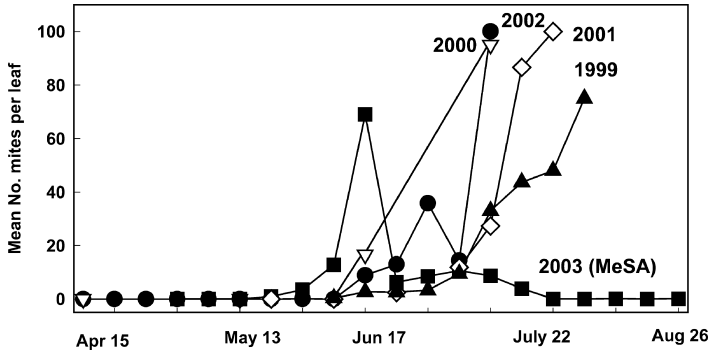

FIG. 8. Weekly mean abundance of spider mites in a methyl salicylate-treated hop yard in 2003 and in 1999, 2000, 2001, and 2002 when methyl salicylate was not used. Miticides were applied in 1999–2002 at points where plots end. No miticide was used in 2003.

(James et al., 2003) to achieve subthreshold (10 mites/leaf) levels. Predatory mite (Phytoseiidae) numbers at this time were low (~0.2 mites/leaf) and could not have been responsible alone for the dramatic decline in spider mites. The most abundant spider mite predators in the yard at this time were *O. tristicolor*, lacewings, *G. pallens*, and *D. brevis*. It is likely that predation by this assemblage of natural enemies resulted in the observed rapid mite suppression. By early July, large numbers of *S. punctum picipes* also appeared, further strengthening the spider mite predator guild. The great abundance of *S. punctum picipes* in the yard during July when only low numbers of spider mites were present was remarkable. *Stethorus* spp., including S. *punctum picipes,* are specialist spider mite predators (Chazeau, 1985), and we speculate that these beetles were transitory in the yard, flying in, staying briefly, then flying out again when faced with a limited food resource.

The evidence presented here is suggestive that the use of controlled-release synthetic MeSA in a crop can increase recruitment and residency of populations of certain beneficial insects. The vineyard data are perhaps most compelling because the experiment was replicated, nevertheless, the hop yard results, while more preliminary and equivocal, do provide a strong indication that not only can recruitment and residency of natural enemies be enhanced by MeSA, but biological control may be improved too. In annual crops and perennial crops like hops where complete dieback occurs during winter, spring recruitment of winged insect predators is critical for seasonal establishment of conservation biological control (James et al., 2003). Generalist predators like *O. tristicolor*, *G. pallens*, lacewings, and hover flies, as well as the spider mite specialist, *S. punctum picipes*, are key players in early season biological control of mites and aphids on hops in the Pacific Northwest (James et al., 2003). Recruitment of these predators to hops during spring varies from year to year, and MeSA may provide a way of increasing the reliability and levels of recruitment. Just as importantly, it may ensure populations of predators remain at high levels in the crop despite fluctuations in pest densities.

The attraction of insects from a variety and range of families to MeSA, a single compound found in many HIPV blends, is remarkable. This suggests, perhaps, that the mechanism of attraction may be more complex than simple directional responses to an attractive volatile. MeSA is a plant–plant signaling compound as well as a plant–arthropod signal, and it is possible that our deployment of MeSA among hop and grape vines stimulated plant–plant communication. The presence of artificially higher concentrations of MeSA in the hop yard and vineyard air may have caused plants to modify their direct and/or indirect defenses. Plants exposed to MeSA may activate disease or arthropod resistance mechanisms. Evidence for MeSA as a plant–plant signal for pathogen resistance in tobacco was provided by Shulaev et al. (1997). It is conceivable that the reduced levels of spider mites in the MeSA-treated hop yard were in part caused

by elevated plant resistance or repellence (Dicke, 1986; Dicke and Dijkman, 1992). Alternatively, MeSA may have activated indirect defenses in the plants, resulting in emission of a complex blend of predator-attracting volatiles. Ozawa et al. (2000b) showed that gaseous MeSA elicited release of predator-attracting volatiles in bean leaves similar to that produced by spider mite feeding. Thus, the insects we recorded at higher levels in MeSA-treated grapes and hops may have been responding to a more complex bouquet than MeSA alone. It is also possible that all three mechanisms (direct attraction, direct plant defense, indirect plant defense) or any combination, may have played a role in our experiments. It will be important to determine the mechanism(s) operating, so that exploitation of MeSA in pest management can be optimized. The practical use of synthetic MeSA as a signaling compound for plants, alerting them to produce their optimal HIPV blends for natural enemy recruitment, would be a particularly useful strategy. Research into using another plant–plant signal, methyl jasmonate, is already well advanced and showing potential as a strategy for both mediating plant resistance (Thaler et al., 2001) and increasing populations of natural enemies (Thaler, 1999, 2002).

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RESPONSES OF *Teretrius nigrescens* TOWARD THE DUST AND FRASS OF ITS PREY, *Prostephanus truncatus*

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Abstract—*Teretrius nigrescens* is considered to be a specialized predator of *Prostephanus truncatus*, a serious pest of stored maize and dried cassava roots. Using a bait-bag behavioral bioassay, this investigation found *T. nigrescens* to be strongly arrested by dust and frass produced by *P. truncatus* on maize, whereas responses to maize flour were weak. Attempts to increase the arrestiveness of flour by altering its physical properties (coarseness or particle size range) were unsuccessful. The arrestive property of dust/frass did not degrade with long-term storage nor did it volatilize or degrade with oven baking up to 150°C. However, extraction with methanol and reapplication was successful in transferring the arrestive property from the dust/frass onto maize flour. The prey dust/frass also induced more oviposition than did maize flour, as judged by production of F1 offspring. The results provide strong evidence for the existence of a high boiling point compound(s) in *P. truncatus* dust/frass that acts as a contact kairomone for *T. nigrescens*. This, or another compound(s) also seems to act as an oviposition stimulant for female *T. nigrescens*.

Key Words—*Teretrius nigrescens*, *Prostephanus truncatus*, Coleoptera, Bostrichidae, Histeridae, predator, contact kairomone, involatile kairomone, arrestant, arrestment, oviposition stimulant.

INTRODUCTION

Prostephanus truncatus (Horn) (Coleoptera: Bostrichidae) is an important pest of stored maize that produces large quantities of dust and frass by boring. It is indigenous to Mesoamerica (Wright, 1984), but in the late 1970s was accidentally

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imported into East Africa where a serious outbreak was reported (Dunstan and Magazini, 1981). Two other accidental introductions followed in West Africa (Harnisch and Krall, 1984; Kalivogui and Mück, 1991). Since then, *P. truncatus* has spread, and its presence has so far been confirmed in 16 African countries (Farrell, 2000). In 1991, the predator *Teretrius nigrescens* (Lewis) (Coleoptera: Histeridae) (formerly *Teretriosoma nigrescens*; see Mazur, 1997) was introduced from the Americas into Africa as a potential classical biological control agent of *P. truncatus* (Richter et al., 1998).

T. nigrescens is believed to be a specialized predator in natural situations. It can detect the *P. truncatus* aggregation pheromone (Scholz et al., 1998), and flying beetles can be attracted to this kairomone from considerable distances (Rees et al., 1990; Böye et al., 1992; Helbig et al., 1992; Key et al., 1994; Scholz et al., 1998). However, what happens at close range, for example inside an infested maize store, is still largely unknown (Rees, 1990). In the laboratory, limited reproduction of *T. nigrescens* has been recorded on monocultures of seven different species of stored product Coleoptera, in addition to *P. truncatus* (Rees, 1991, 1992; Pöschko, 1994; Helbig, 1995). However, development on these other species tested was poor, and it seems that *T. nigrescens* is relatively specific in its requirements for successful development. Hence, it is likely that *T. nigrescens* may have evolved specialized behaviors for the location of *P. truncatus* in addition to the response to the aggregation pheromone.

In another beetle family, the Scolytidae, predators of economically important species have been well studied, and many show a kairomonal response to prey aggregation pheromone (Dyer, 1975; Billings and Cameron, 1984; Mendel, 1988). In contrast, predators such as *Rhizophagus grandis* (Coleoptera: Rhizophagidae) that consume nonaggregating Scolytidae exploit plant-derived volatiles in prey frass as long-range cues (Wainhouse et al., 1991, 1992; Gregoire et al., 1992; Wyatt et al., 1993). The same frass volatiles at close range also increase the oviposition rate of *R. grandis* (Gregoire et al., 1991).

This paper reports a series of bioassays exploring the possibility that the dust and/or frass produced by *P. truncatus* provides a source of cues exploited by *T. nigrescens* in close-range location of its prey. Results presented also provide insights into the nature of the cue(s).

METHODS AND MATERIALS

Maize and Insects. Organically grown, yellow dent maize (Gillit and Cook Ltd., Faversham, UK) was used for all experiments, culturing, and flour production. The *P. truncatus* and *T. nigrescens* used were originally obtained from the Volta Region of Ghana, West Africa in 1999. Before use in bioassays, *T. nigrescens* were preconditioned. The dust was cleaned off of them by sprinkling with water

and they were dried with paper towel, before being stored for 24 hr in sets of 10 in a controlled temperature and humidity (CTH) room (27 ± 2°C; 60 ± 5% RH; 12L:12D photoregime). Despite efforts, no way was found to determine the sex of *T. nigrescens* without killing them, so mixed sex samples were always used.

Bait-Bag Behavioral Bioassay. Behavioral responses of *T. nigrescens* were assessed by a bioassay in which treatments were presented in small-mesh pyramidal "bait-bags" located in the center of a jar filled with maize. Bait-bags were constructed from rectangular pieces of mesh (Netlon^{TM}, 2 mm; 16 × 8 cm). The rectangle was folded in half to form a square, and the parallel open sides were stapled. A length of thin polypropylene twine was tied into this open bait-bag to aid removal at the end of the experiment. The experimental treatment (5 g) was mixed in a beaker with clean maize grains (35 g) before adding to the bait bag. Responses to maize grains alone (40 g) were also assessed. The open side of the bait-bag was closed by stapling twice.

For each assay, clean maize (300 g) was poured into a glass jar (2 l). The filled bait-bag was then positioned on the center point of this basal layer and another portion of maize (700 g) was carefully added on top. Preconditioned adult *T. nigrescens* (10) were placed into each jar, and the jars were sealed with filter paper and wax and stored in the CTH room. For any given experiment, all bioassay treatments and replicates were tested simultaneously, and for each treatment there were always five replicates.

The experiments lasted 3 days and were started and ended at about midday. The jars were opened while still in the CTH room, and the bait-bags were carefully removed using the string and placed individually into labeled dishes. They were then opened in turn and, the number of *T. nigrescens* within each was found by sieving, pausing, and re-sieving three times (0.5 cm and 850 μm sieves; Endecotts, London, UK).

Preparation of Dust/Frass and Maize Flour. Cultures of *P. truncatus* were started with unsexed insects (200) on maize (1 kg), and jars were stored in CTH rooms for 8 wk. Bored maize grains and insects were then separated from the dust and frass (hereafter referred to as dust/frass) by shaking through a sieve (500 μm) by hand. In one experiment, dust/frass was obtained from a culture started with both *P. truncatus* (200) and *T. nigrescens* (10). All dust/frass was frozen (−20 ± 2°C) for at least 7 days before use in bioassays.

Maize flour was produced by passing whole grains slowly once through a hammer-mill (Glen Creston DFH 48) operating at maximum speed. The milled product was sieved to separate the flour (<500 μm) from the residue (>500 μm). This sieved flour was frozen for at least 7 days before use as above.

Dust/frass originating from *P. truncatus* cultures on maize is uniform with over 80% by mass of particles having diameters of 125–149 μm, while maize flour prepared as above contains 40% by mass of particles having 125–149 μm diameters (Stewart-Jones, 2002). Although the other particles present in the maize

flour give it a slightly coarser overall texture than dust/frass, it was considered to be a reasonable approximation (Stewart-Jones, 2002). Re-milling of the original milled product actually resulted in increasingly coarse flours because more of the hard crystalline parts of the grain were breaking down to just below 500 μm. In one experiment, maize grains or milled product were passed through the mill 1–5 times to produce increasingly coarse flours, and these were tested in the bait-bag bioassay. In another experiment, samples of dust/frass or maize flour were divided into two, and one half was hand shaken through 150- and 125-μm sieves to give material with particle size ranges of 125–149 μm only. The other half of each was kept unsieved as a control, and responses to uniform or control samples were tested in the bait-bag bioassay.

Bioassay of Dust/Frass and Flour after Varying Periods of Storage. Samples of dust/frass and flour were maintained in jars sealed with filter-paper in a CTH room (27 $\pm$ 2°C; 60 $\pm$ 5% RH) where gradual change in the material might be expected. Equivalent samples of dust/frass and flour were stored in a deep freeze (-20 ± 2°C) where minimal change would be expected. Bait-bag bioassays were undertaken on eight occasions over a 48-wk period, and responses allowed comparison of the differently stored materials. Each bioassay set required 25 g of material per treatment and since each *P. truncatus* culture yielded around 250 g of dust/frass, it was necessary to mix material from two cultures in order to have sufficient material to run the experiment. Dust/frass originating from two different cultures was shaken vigorously in a clean plastic bag and divided into two halves, which were subjected to the different storage treatments. Two samples of maize flour (250 g) were also subjected to the two storage treatments, and samples of these were bioassayed at the same time as the dust/frass.

Heat Treatment of Dust/Frass and Flour. To test the effects of heating dust/frass or flour on the response of *T. nigrescens*, samples were baked in an oven. The technique used was a modification of the standard method for determining moisture content (e.g., AOAC, 1984; AACC, 1990). Five samples of dust/frass (35 g) and five samples of flour (35 g) were prepared and spread thinly on metal trays and heated in fan ovens for 3 hr at temperatures of 50, 100, 150, and 200°C. After heating, samples were placed in jars sealed with filter paper and stored in a CTH room with regular shaking for 2 wk to equilibrate before bioassaying. An unheated sample of both materials was maintained in the CTH room as control.

Chemical Extraction from Dust/Frass and Reapplication onto Flour. To investigate whether the component(s) in dust/frass to which *T. nigrescens* responds could be extracted with a solvent, bioassays were carried out with dust/frass samples after extraction with methanol and flour samples to which the extract had been reapplied. A sample of dust/frass (60 g) was divided equally. One subsample was kept as a control and the other was placed in a cellulose extraction thimble (height 123 mm, diam. 41 mm; Whatman) and Soxhlet extracted for 24 hr with methanol (300 ml) cycling about once every 40 min. A sample of maize flour (30 g) was

extracted in the same manner. After extraction, the remaining solvent was allowed to evaporate overnight from the thimbles in a fume cupboard.

To reapply the dust/frass extract, all of the methanol extract was mixed with clean maize flour (30 g) in a round-bottomed flask, and the solvent was evaporated using a rotary evaporator at 35°C. To control for any solvent effect, methanol (300 ml) was added to maize flour (30 g), and the solvent was evaporated in the same manner. Having evaporated most of the solvent, the flour was left in a fume cupboard overnight and then shaken through a sieve (500 μm) to break up any lumps that had formed.

Oviposition Bioassay. The effects of *P. truncatus* dust/frass on oviposition by *T. nigrescens* were measured in terms of numbers of resulting *T. nigrescens* F1 adults, since these could be easily counted. Maize only (350 g), maize (300 g) mixed with of flour (50 g), or maize (300 g) mixed with dust/frass (50 g) were placed in jars (0.5 l). Conditioned *T. nigrescens* (10) were placed in each jar, and there were five replicates of each treatment. As *T. nigrescens* cannot be sexed alive, some of the jars would not have contained a 50:50 sex ratio, but it was hoped that having five replicates would minimize the effects of this experimental limitation.

The jars were left for 7 days in a CTH room, after which the contents were poured out onto a metal tray and carefully sifted through until all of the adult *T. nigrescens* were recovered. All of the remaining material was returned to the jar, and adult *P. truncatus* (50) were added to each jar before sealing and storing in a CTH room for 8 wk. The jars were then opened and thoroughly sieved. Although 8 wk should be long enough to ensure development from egg to adult (Rees, 1985; Leliveldt and Laborius, 1990; Oussou et al., 1999), large numbers of last instar *T. nigrescens* larvae were observed in some of the treatments. It was unclear whether these were F1 larvae that were still developing or the F2 generation. The early instar larvae seen were assumed to be F2. Thus the numbers of both *T. nigrescens* adults and apparent last instars recovered were recorded separately. Since *T. nigrescens* has only two larval instars, last instars were easily identified (Rees, 1985).

Data Analysis. Data were not distributed normally and were consequently analyzed using nonparametric statistics. A Kruskall–Wallis test was used to determine whether or not there was significant heterogeneity between multiple treatments, and Mann–Whitney tests were used to compare paired treatments. Differences were considered to be significant if the probability of their occurrence by chance was less than 5%.

RESULTS

Response of T. nigrescens to Maize Flour or P. truncatus Dust/Frass. In the bait-bag bioassay, more *T. nigrescens* were found in bait-bags containing

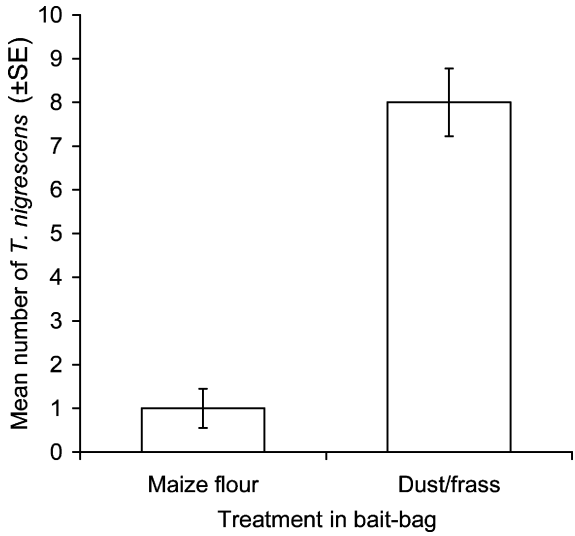

FIG. 1. Mean (±SE) number of *T. nigrescens* recovered from bait-bags containing maize flour or *P. truncatus* dust/frass ($N = 5$).

P. truncatus dust/frass than in those containing maize flour (Figure 1; Mann–Whitney: $N = 5$, $W = 15.0$, $P = 0.012$).

Response of T. nigrescens to Dust/Frass from P. truncatus Culture or a Mixed P. truncatus/T. nigrescens Culture. In the bait-bag bioassay, the response of *T. nigrescens* was stronger to either *P. truncatus* dust/frass (Mann–Whitney $N = 5$, $W = 15.0$, $P = 0.012$) or *P. truncatus/T. nigrescens* dust/frass (Mann–Whitney $N = 5$, $W = 15.5$, $P = 0.016$) than to maize flour (Figure 2). However, response to the two types of dust/frass appears to have been equally strong (Figure 2; Mann–Whitney: $N = 5$, $W = 32.5$, $P = 0.347$).

Response of T. nigrescens to P. truncatus Dust/Frass Originating from Replicate Sources. When dust/frass originating from five different *P. truncatus* cultures and flour were bioassayed, there was no significant heterogeneity in *T. nigrescens* responses to the different dust/frass samples (Figure 3; Kruskall–Wallis across all dust/frass: $h = 3.44$, $df = 4$, $P = 0.487$).

Response of T. nigrescens to Maize Flours Milled to Different Degrees. There were no differences in numbers of *T. nigrescens* found in bait-bags containing maize flours subjected to 1–5 millings (Figure 4; Kruskal-Wallis: $h = 3.24$, $df = 4$, $P = 0.518$). In this experiment, the response to bait-bags containing maize flour was greater than to those containing maize grain only (Figure 4). This suggests that the flour-like properties of dust/frass may contribute, albeit mildly, to its overall arrestive qualities.

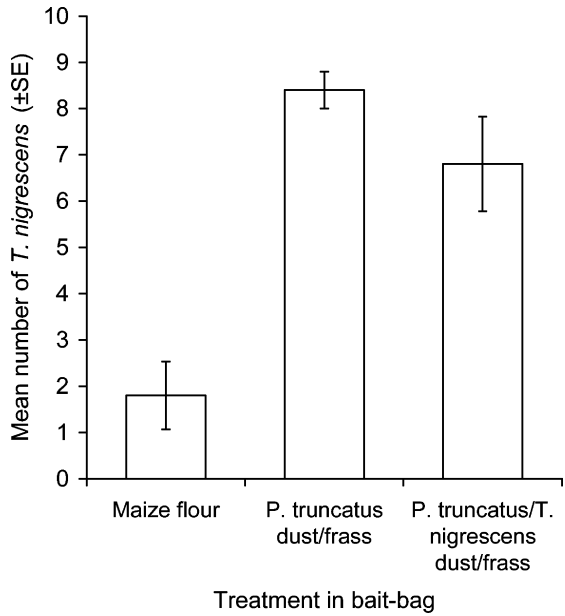

FIG. 2. Mean (±SE) number of *T. nigrescens* recovered from bait-bags containing maize flour, *P. truncatus* dust/frass or *P. truncatus/T. nigrescens* dust/frass ($N = 5$).

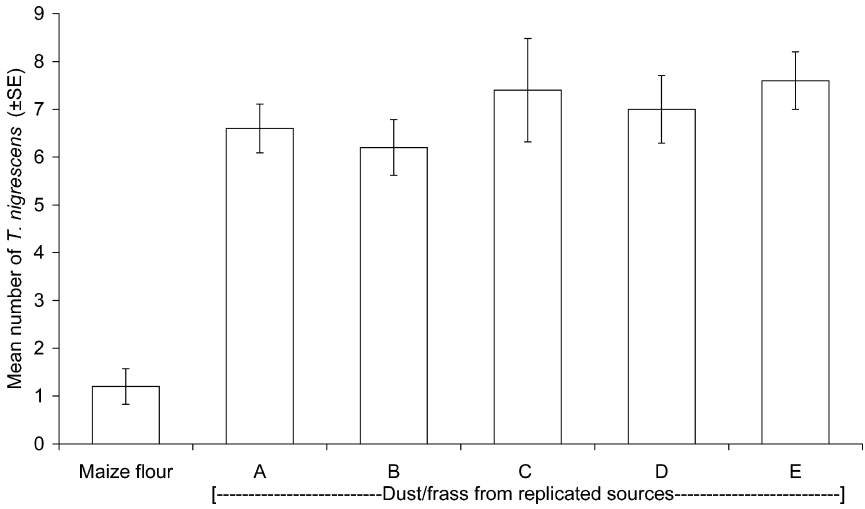

FIG. 3. Mean (±SE) number of *T. nigrescens* recovered from bait-bags containing maize flour or *P. truncatus* dust/frass from five replicated sources ($N = 5$).

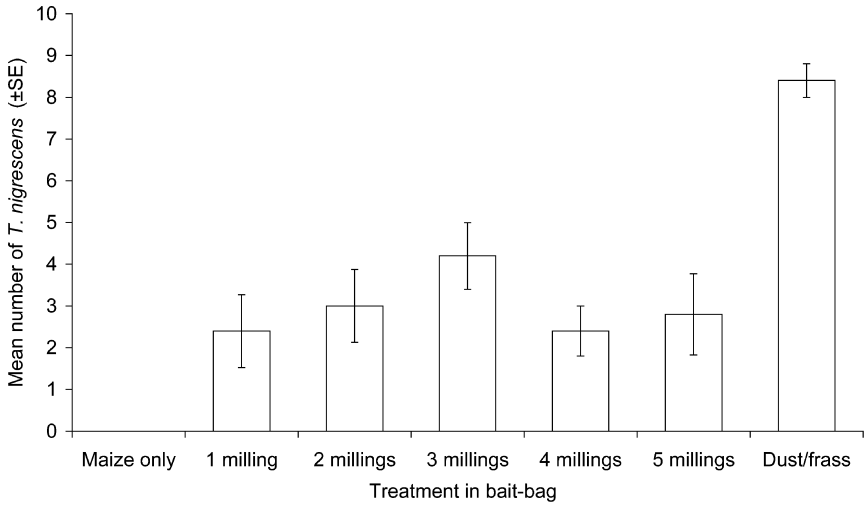

FIG. 4. Mean (±SE) number of *T. nigrescens* recovered from bait-bags containing maize grains, maize flours milled to different degrees or *P. truncatus* dust/frass ($N = 5$).

Response of T. nigrescens to Maize Flour or P. truncatus Dust/Frass with Particle Sizes in the 125–149 μm Range. When *T. nigrescens* was presented with maize flour and dust/frass of identical particle sizes (125–149 μm), numbers in bait-bags containing the dust/frass were still greater than those in bags containing the flour (Figure 5; Mann–Whitney $N = 5$, $W = 15.0$, $P = 0.012$). There were no differences in numbers found in bags containing graded or crude material for either the dust/frass (Mann–Whitney $N = 5$, $W = 27.5$, $P = 1.0$) or the flour (Mann–Whitney $N = 5$, $W = 27.0$, $P = 1.0$). Significantly fewer were found in bags containing only maize grain than in bag containing maize mixed with maize flour (Mann–Whitney $N = 5$, $W = 15.0$, $P = 0.012$) or graded flour (Mann–Whitney $N = 5$, $W = 15.5$, $P = 0.016$).

Response of T. nigrescens to P. truncatus Dust/Frass Stored for Periods up to 48 wk Either Frozen or Under Ambient Conditions. When responses of *T. nigrescens* to bait-bags containing dust/frass that had been stored in the CTH room or in a freezer were compared at intervals over 48 wk, no significant differences in numbers were recorded on any sampling occasion (Figure 6, Mann–Whitney all $P \geq 0.05$). There were differences in response to dust/frass and flour stored in the CTH room on all occasions, except for wk 4 (wk 4: $N = 5$, $W = 21.0$, $P = 0.210$; all others $P \leq 0.05$). However, to the human nose, the initial characteristic odor of dust/frass maintained in the CTH room declined to odorless as weeks proceeded, whereas frozen dust/frass kept its characteristic odor. The intensity of the responses to all dust/frass treatments (either frozen or CTH) tended to go up

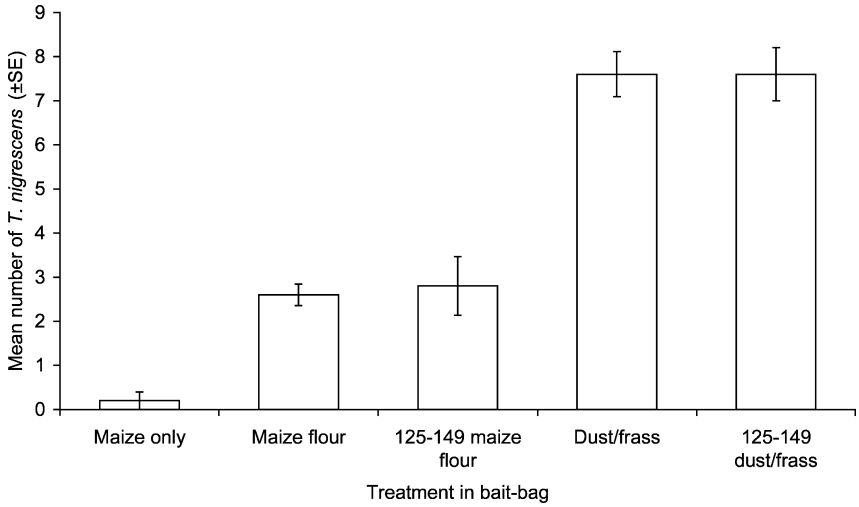

FIG. 5. Mean (±SE) number of *T. nigrescens* recovered in bioassays from bait-bags containing maize flour or *P. truncatus* dust/frass both pre-sieved so that all particles fall within the 125–149 μm range ($N = 5$).

and down together depending on sampling occasion (Figure 6). This might be explained by previous culturing conditions or uncontrolled external factors such as changes in atmospheric pressure.

Response of T. nigrescens to P. truncatus Dust/Frass After Heat Treatment. Samples of both dust/frass and maize flour were subjected to a range of heat treatments. There was significant heterogeneity in the response of *T. nigrescens* to the dust/frass treatments (Figure 7; Kruskall–Wallis all dust/frass: $df = 4$, $h = 9.67$, $P = 0.046$), but not for the flour treatments (Kruskall–Wallis all flour: $df = 4$, $h = 3.53$, $P = 0.467$). The 200°C treatment was visibly different from the other treatments, being burnt and coffee-colored. On reanalysis of the data that omitted results for this treatment, there was no significant heterogeneity of response (Kruskall–Wallis over CTH, 50, 100, and 150°C dust/frass: $df = 3$, $h = 0.847$, $P = 0.828$). With the exception of the 200°C pretreatment, responses to dust/frass were all stronger than to correspondingly treated flours (200°C: $N = 5$, $W = 20.5$, $P = 0.174$; all others $P \leq 0.05$).

Response of T. nigrescens to P. truncatus Dust/Frass After Chemical Extraction and Reapplication. Numbers of *T. nigrescens* found in bait-bags containing dust/frass after Soxhlet extraction with methanol were lower than found in bags containing untreated dust/frass, but this just failed to be statistically significant (Figure 8 and Table 1; $P = 0.06$). However, *T. nigrescens* response to the dust/frass after extraction was not different from that to maize flour (Table 1;

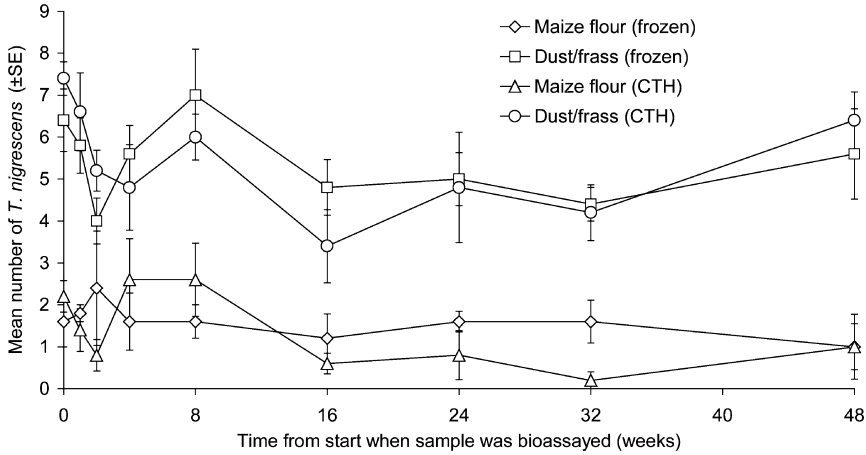

FIG. 6. Mean (±SE) number of *T. nigrescens* recovered from bait-bags containing stored samples of maize flour or *P. truncatus* dust/frass ($N = 5$).

$P = 0.144$). Moreover, more *T. nigrescens* were found in the bait-bags containing maize flour treated with the extract of dust/frass than in those containing untreated flour (Table 1; $P = 0.028$), and there was no difference in response between maize flour treated with extract and untreated dust/frass (Table 1; $P = 0.296$).

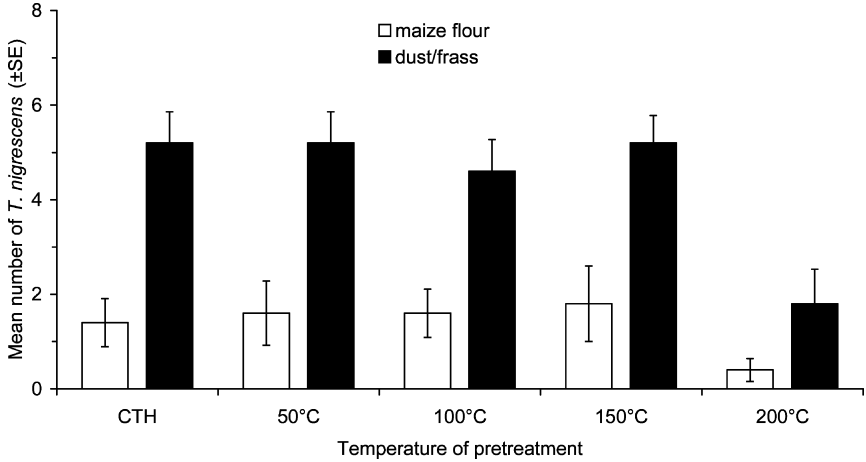

FIG. 7. Mean (±SE) number of *T. nigrescens* recovered from bait-bags containing maize flour or *P. truncatus* dust/frass that had been previously heated to a range of temperatures ($N = 5$).

FIG. 8. Mean (±SE) number of *T. nigrescens* recovered from bait-bags containing maize flour, *P. truncatus* dust/frass, samples that had been subjected to solvent extraction or flour impregnated with the extract ($N = 5$).

Effect of P. truncatus Dust/Frass on Oviposition by T. nigrescens. When *T. nigrescens* were allowed to mate and oviposit in maize, maize mixed with maize flour, or maize mixed with *P. truncatus* dust/frass, there was significant heterogeneity between the treatments in the numbers of recovered F1 adults (Kruskall–Wallis $N = 5$, $h = 10.59$, $P = 0.005$) and larvae (Kruskall–Wallis $N = 5$, $h = 9.38$, $P = 0.009$) (Figure 9). More of both stages were found in maize mixed with dust/frass than in maize mixed with maize flour only (Mann–Whitney maize flour vs. dust/frass: adults $N = 5$, $W = 16.5$, $P = 0.028$; larvae $N = 5$, $W = 17.0$, $P = 0.037$).

TABLE 1. SUMMARY OF ANALYSIS COMPARING MAIZE FLOUR, DUST/FRASS, AND SAMPLES THAT HAD BEEN SUBJECTED TO SOLVENT EXTRACTION OR IMPREGNATION USING THE EXTRACT*

Treatments being compared	*W*	*P*-value
Dust/frass vs. dust/frass Soxhlet extracted	18.0	0.060
Dust/frass Soxhlet extracted vs. maize flour	20.0	0.144
Maize flour + dust/frass extract vs. maize flour	16.5	0.028
Maize flour + dust/frass extract vs. dust/frass	22.0	0.296

*Mann-Whitney; $N = 5$ for all.

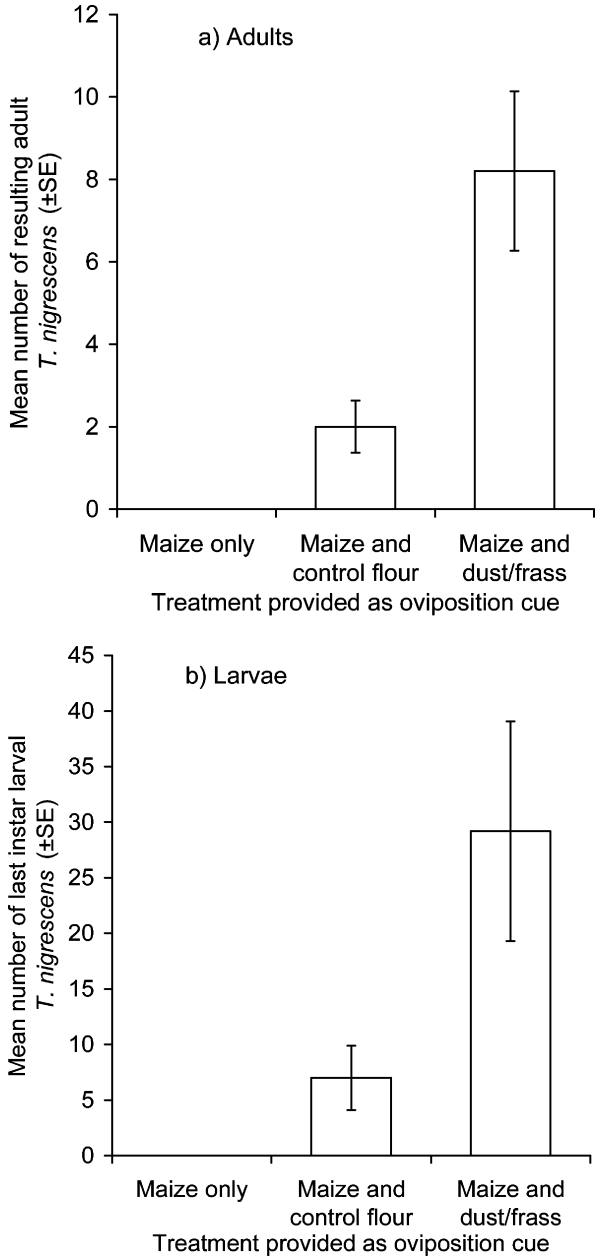

FIG. 9. Number of F1 adults and late instar larvae recovered by sieving after parent *T. nigrescens* were presented with different experimental habitat treatments ($N = 5$).

DISCUSSION

The results provide strong evidence for the existence of a compound(s) of high boiling point in *P. truncatus* dust/frass that acts as a contact kairomone for adult *T. nigrescens*. This or another compound(s) also seems to act as an oviposition stimulant for female *T. nigrescens*.

In the aphids, honeydew is generally thought of as contact kairomone that arrests aphidophagous predators. However, there is some debate over how much of the arrestive response results from feeding on this sugary excretion (Carter and Dixon, 1984; Heidari and Copland, 1993). Similarly, it might be argued that the arrestive response of *T. nigrescens* to dust/frass is due to a scavenging effect, possibly because of the presence of eggs or other scavengeable materials. Certainly, there would have been no live *P. truncatus* material in the bioassayed dust/frass since it had been previously frozen. Shires (1980) noted that *P. truncatus* eggs, were retained by a 250-μm sieve, and the above experiment in which flour and dust/frass were both sieved to 125–149 μm, not only confirmed that exact flour physical properties were unimportant in the response, but also would have excluded intact eggs as a significant explanatory factor. Observation of dust/frass using a microscope, both before and after freezing, did not show any eggs, although the dust was seen to be heavily speckled with large amounts of *P. truncatus* excrement. Nevertheless, it should be assumed that dust/frass contamination by eggshells and other residues from eggs is almost inevitable. The experiment demonstrating that the effect could be transferred from dust/frass onto flour using methanol not only provided strong evidence that a chemical(s) is causing the response, but also excluded any possible scavenging effects. It should be noted that certain chemical changes, for example methylation, might have occurred during this extraction process. The applied extract was found to be bioactive, although it may have contained some compounds that were not actually present in normal dust/frass.

In addition to causing arrestive responses in *T. nigrescens*, the results suggest that dust/frass acts as an oviposition stimulant. This conclusion can only be accepted tentatively as it is based on counts of F1 progeny and not recordings of actual oviposition. A less likely alternative explanation could be that parental *T. nigrescens* laid similar numbers of eggs in all the treatments but that the presence of dust/frass improved egg and/or larval survival.

Attraction of predators to volatile kairomones is well documented. Predators of scolytids, for example, respond to prey specific pheromones and/or compounds in the frass that originate from prey diet (Billings and Cameron, 1984; Miller et al., 1987; Wainhouse et al., 1991; Seybold et al., 1992; Royer and Boivin, 1999). There is no doubt that *T. nigrescens* responds to *P. truncatus* aggregation pheromone in flight (Rees et al., 1990; Böye et al., 1992), but it is probable that it is not used at close range. Although Key et al. (1994) noted a *T. nigrescens* walking

directly towards a pheromone lure in the field, Hodges and Dobson (1998) failed to detect any response to synthetic pheromone in a walking laboratory bioassay. Experiments presented above, in which dust/frass was stored or heated, were presumed to have removed any traces of aggregation pheromone components or similarly volatile materials that might have been in the dust/frass. Indeed, other recent research has demonstrated that *T. nigrescens* is actually repelled, when walking, by trace quantities of *P. truncatus* pheromone (Stewart-Jones, 2002). Thus, it can be concluded that chemical cues of high boiling point in the dust/frass are causing the arrestive response. With regard to volatiles in prey diet, *T. nigrescens* responses to maize, cassava, and to a lesser extent wheat have been reported by Rees (1992). However, responses were compared against "Hortag," an inert synthetic material that made the bioassay unnatural. Similarly, findings presented here in which responses to maize grain mixed with flour were higher than to maize grain alone suggest some kind of response to maize flour or dustiness itself. However, this is unlikely to be due to volatiles from the flour since the response did not change with storage or heating.

Contact kairomones are important cues used in parasitoid foraging. Such chemical cues of high boiling point can result in arrestive responses by increasing the total time of random search in an area (Waage, 1978) and are commonly important in host assessment prior to oviposition (Vinson, 1985, 1991). Recently, it was demonstrated that a parasitoid of rose hip flies gains directional information from host contact kairomones, and this leads to directed search (Hoffmeister et al., 2000). By contrast, a search of the literature that excluded responses of aphidophagous predators, only managed to identify one example of a predator using contact kairomones. This one study found waxes from coccid prey to have an oviposition stimulating effect on the coccinellid *Cryptolaemus montrouzieri* (Merlin et al., 1996). The current paper is, therefore, the second study to report use of contact kairomones in non-aphidophagous predators. Obviously, responses such as those of *C. montrouzieri* and *T. nigrescens* are biologically advantageous so similar responses might actually be quite widespread among predators, particularly specialists. Perhaps this lack of documentation for the role of contact kairomones in predators is in part due to their often more generalist nature when compared with parasitoids and their hosts. Furthermore, the complicated sequence of parasitoid behaviors leading to acceptance and finally oviposition has attracted greater attention and has hence contributed to this imbalance. Nevertheless, as for parasitoids, appropriate responses by specialist predators to any precontact cue(s) of high boiling point, for example the presence of prey epicuticular waxes, would be selected for.

Large *P. truncatus* populations, such as those found in stores, are likely to be recent phenomena, and the investigated *T. nigrescens* prey location mechanisms are more likely to have evolved for locating widespread and sparse populations of *P. truncatus* boring into wood of probably a number of different species

(Hodges et al., 1999). Bearing this in mind, it is not surprising that *T. nigrescens* do not appear to utilize chemical cues associated with the substrate into which *P. truncatus* is boring as these cues would be unreliable. We can now summarize *T. nigrescens* prey location in the light of new findings presented here. Flying *T. nigrescens* locate suitable habitats such as infested maize stores by exploiting *P. truncatus* aggregation pheromone (Rees et al., 1990; Böye et al., 1992). Within the natural habitat or store, pheromone is not exploited as a close range attractive cue (Hodges and Dobson, 1998; Stewart-Jones, 2002), and chemical cue(s) of high boiling point in *P. truncatus* dust/frass arrest *T. nigrescens* in areas in which prey presence is likely. The dust/frass also appears to stimulate oviposition in females. Presumably, contact with actual food items within the patch is by random search, as no directional information has been implicated so far.

The principal *T. nigrescens* response described in this work was arrestment. Using the current bioassay design, no behavioral observations could be made and the behavioral mechanisms involved in this response could not be elucidated. Further work on analysis of the behavioral mechanisms as well as the origin and chemical nature of this cue(s) will be reported in subsequent papers.

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IDENTIFICATION AND QUANTITATION OF COMPOUNDS IN A SERIES OF ALLELOPATHIC AND NON-ALLELOPATHIC RICE ROOT EXUDATES

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Abstract—An investigation of the chemical basis for rice allelopathy to the rice weed arrowhead (*Sagittaria montevidensis*) was undertaken using GC/MS and GC/MS/MS techniques. Twenty-five compounds were isolated and identified from the root exudates of both allelopathic and non-allelopathic rice varieties. Phenolics, phenylalkanoic acids, and indoles were among the chemical classes identified. Two indoles previously unreported in rice were detected in the exudates, 5-hydroxy-2-indolecarboxylic acid and 5-hydroxyindole-3-acetic acid. Several other compounds identified in this study have not previously been reported in rice root exudates, namely mercaptoacetic acid, 4-hydroxyphenylacetic acid, and 4-vinylphenol. The levels of 15 compounds present in the exudates were quantified using GC/MS/MS. Six of the seven most abundant compounds were phenolic acids. Significant differences exist between the allelopathic and non-allelopathic cultivars in their production of three of these six compounds. Greater amounts of *trans*-ferulic acid, *p*-hydroxybenzoic acid, and caffeic acid were detected in the exudates of allelopathic cultivars. The seventh compound, abietic acid, was significantly higher in the non-allelopathic cultivars.

Key Words—Allelochemicals, hydroxamic acids, hydroxystyrenes, phenolic acids, quantitation, rice, root exudates, *Sagittaria montevidensis*, vinylphenols, weed suppression.

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INTRODUCTION

Although most plant tissues contain potential allelochemicals (Putnam and Tang, 1986), only those compounds released from the plant into the environment are available to exert an allelopathic effect on another organism. Wu et al. (2001) found that there was no significant correlation between the level of allelochemicals detected within wheat roots and the level in wheat root exudates. For allelopathic studies, root exudate measurements are more biologically meaningful than tissue content (Perez et al., 1991; Niemeyer et al., 1995), but they are more difficult to conduct as the responsible compounds are found in such small quantities (Bouillant et al., 1994) within complex matrices. So far, relatively few studies have focused on plant root exudate rather than plant extracts.

Analysis of such small amounts of chemicals has become easier and more cost-effective owing to the development of certain hyphenated chromatographic techniques such as GC or LC/MS. GC/MS analysis can identify pure compounds present at less than 1 ng (Liebler et al., 1996). GC/MS/MS is superior to GC/MS for the analysis of complex mixtures owing to its enhanced selectivity and sensitivity (Durant et al., 2002). MS/MS is preferential for low molecular weight molecules ($<$1000 amu) in complex mixtures due to its high specificity for target analytes and decreased susceptibility to mass interferences (Van Pelt et al., 1998). The power of GC/MS/MS techniques to analyze complex mixtures such as those found in plant root exudates has been gaining attention. Wu et al. (1999, 2000, 2001) used GC/MS/MS analysis to determine the composition of wheat root exudates and quantitate the phenolic acids and hydroxamic acids present at very low concentrations (μg/l).

To date, several different chemical classes have been implicated as allelochemicals in rice allelopathy, including indoles, phenylalkanoic acids, terpenoids, fatty acids, hydroxamic acids, and phenolic acids (Chou et al., 1991; Mattice et al., 1998; Kato-Noguchi and Ino, 2001; Kong et al., 2002). Chou and Lin (1976) have identified several phenolics in decomposing rice straw. Kim and Shin (1998) isolated fatty acid esters, unsaturated ketones, polycyclic aromatic compounds, and alkaloids from the ethyl acetate fraction of rice extracts during GC/MS analysis. This fraction was shown to be phytotoxic towards barnyardgrass. However, no studies have shown that these chemicals are actually released by living rice plants.

In this study, GC/MS/MS techniques were employed in the identification and quantitation of a range of compounds within complex mixtures of rice root exudates.

METHODS AND MATERIALS

Instrumentation. A Varian 3400 CX gas chromatograph was fitted with a DB-5-MS ITD (30 m $\times$ 0.25 mm $\times$ 0.25 μm) fused silica capillary column obtained

from J&W Scientific (Alltech) and coupled with a Varian Saturn 2000 ion trap-mass spectrometer. Instrument conditions were similar to those used by Wu et al. (1999). The injector temperature was 280°C. The initial column temperature was a constant 80°C for 1 min, increased to 160°C at a rate of 10°C/min, then ramped to 235°C at 5°C/min. The temperature was then brought to 280°C at a rate of 50°C/min and held until the end of the 29.90-min run. Helium was the carrier gas with linear velocity 34 cm/s.

For GC/MS analysis, all pure compounds and root exudates were converted to volatile trimethylsilyl derivatives with BSTFA, and a 4.1-min filament delay was employed to avoid overloading the detector with solvent. The mass scan range was set at 50–650 m/z in the 70 eV electron impact ionization (EI) mode. For GC/MS/MS analysis, the run was divided into 22 acquisition segments for the first method file (Table 1) and 15 segments for the second method file (Table 2). MS/MS parameters such as the excitation amplitude (volts) were optimized for each segment to reflect the nature of the selected potential allelochemicals. All data were analyzed using the Varian (Walnut Creek, California) Saturn Chromatography Work Station software (Version 1.3).

GC/MS and GC/MS/MS Spectral Library Construction. From the limited amount of literature available concerning the composition of rice root exudates, 32 secondary metabolites were selected as potential allelochemicals. All compounds were purchased as pure reference compounds from Sigma–Aldrich (Milwaukee, USA) except for 4-vinylphenol which was supplied by Lancaster Synthesis (Morecombe, UK). These compounds were used to obtain the retention time (rT), the mass spectrum and the daughter mass spectrum (MS/MS) of each compound as authentic reference data.

Because of overlapping retention times, only 20 of the above compounds were selected for quantitation purposes. Selection of MS/MS precursor ions was made on the basis of both high abundance and a mass to charge ratio that was close to that of the molecular ion. The chromatographic run was divided into 22 acquisition time segments with corresponding excitation amplitudes (Table 1). The excitation storage level ($m/z = 48$), excitation time (20 ms), and mass isolation window (3 m/z) remained the same for all acquisition segments.

The retention time (rT) and daughter mass spectra of 11 additional compounds were used to develop a second MS/MS method file (Table 2) solely for identification purposes. Only three allelopathic (Hungarian #1, IET 1444, and Giza 176) and two nonallelopathic varieties (Woo Co Chin Yu and Basmati) were analyzed for this method file. In this series, the excitation storage level was adjusted from 48 to 55 m/z for one compound, α-resorcylic acid. These additional compounds were not included in the quantitation analysis. The mass spectra and the retention times of the TMS-derivatized reference compounds were used for identification of compounds found in the silylated exudates of allelopathic and nonallelopathic rice varieties.

TABLE 1. ION-TRAP MS/MS METHOD FILE 1 PARAMETERS FOR QUANTITATION (REFERENCE COMPOUNDS)

Segment[a]	Compound[b]	rT (min)	Segment range (min)	Precursor ion (m/z)	Excitation amplitude (V)	Quantitation ion (m/z)
2	Mercaptoacetic acid	6.69	6–7.5	221	31.0	147
3	Resorcinol	8.49	7.5–8.7	239	31.9	195
4	*p*-Chlorobenzoic acid[c]	8.91	8.7–9.5	213	34.4	169
5	Salicylic acid	10.28	9.5–10.4	267	29.5	209
6	4-Phenylbutyric acid	10.58	10.4–10.8	221	33.5	203
7	*t*-Cinnamic acid	10.93	10.8–11	205	33.3	159
8	2-Hydroxyphenylacetic acid	11.16	11–11.3	253	30.3	209
9	α-Hydroxybenzenepropanoic acid	11.43	11.3–11.9	267	26.0	235
10	*p*-Hydroxybenzoic acid	12.16	11.9–12.3	267	28.5	223
11	4-Hydroxyphenylacetic acid	12.36	12.3–14	252	27.5	164
12	Vanillic acid	14.51	14–15	297	25.5	267
13	β-Resorcylic acid	15.38	15–15.8	355	21.9	281
14	Indole-3-carboxaldehyde	16.25	15.8–16.6	217	34.7	216
15	Syringic acid	16.93	16.6–17.4	297	26.0	253
16	*p*-Coumaric acid	17.71	17.4–17.8	293	26.25	249
17	Gallic acid	18.01	17.8–19.4	458	15.9	443
18	Indole-5-carboxylic acid	19.98	19.4–20.30	290	26.6	246
19	*t*-Ferulic acid	20.51	20.3–21	338	22.7	323
20	Caffeic acid	21.33	21–22	396	18.2	396
21	5-Hydroxyindole-3-acetic acid	22.68	22–24.8	407	17.8	407
22	Abietic acid	25.33	24.8–29.90	256	27.5	241

[a] Segment 1 was the solvent delay.
[b] Identified as trimethylsilyl derivatives (TMS).
[c] Internal standard.

TABLE 2. ION-TRAP MS/MS METHOD FILE 2 PARAMETERS FOR DETECTION OF COMPOUNDS IN ROOT EXUDATES (REFERENCE COMPOUNDS)

Segment[a]	Compound[b]	rT (min)	Segment range (min)	Precursor ion (m/z)	Excitation amplitude (V)	Excitation storage level (m/z)
2	4-Vinylphenol	6.69	6–8	177	35.5	48
3	*p*-Chlorobenzoic acid[c]	8.46	8–10	213	34.4	48
4	3-Hydroxybenzoic acid	10.65	10–11	267	28.7	48
5	Penicillamine	11.60	11–13	291	25	48
6	3-(4-Hydroxyphenyl)propionic acid	13.81	13–14.2	310	24	48
7	*o*-Coumaric acid	14.63	14.2–14.8	293	26	48
8	α-Resorcylic acid	15.88	14.8–15	370	15	55
9	Umbelliferone	15.13	15–16	219	32	48
10	Hydrocaffeic acid	17.09	16–18	398	67	48
11	Scopoletin	18.68	18–19.2	264	29	48
12	Esculetin	19.84	19.2–20.1	395	23.2	48
13	5-Hydroxy-2-indolecarboxylic acid[d]	20.58	20.1–21	321	26	48
14	5-Hydroxy-2-indolecarboxylic acid	21.64	21–29.9	379	19.4	48

[a] Segment 1 was the solvent delay.
[b] Identified as trimethylsilyl derivatives (TMS).
[c] Internal standard.
[d] Two peaks were detected for 5-hydroxy-2-indolecarboxylic acid.

Collection and Preparation of Rice Root Exudates. The method for rice exudate sample preparation was modified from Wu et al. (1999). Fifteen sterilized pregerminated seeds of allelopathic (Takanenishiki, IET 1444, Giza 176, Hungarian #1) and nonallelopathic rice (Pelde, TN-1, Toro, Woo Co Chin Yu, Basmati) were grown in 30 ml of 0.3% water–agar for 17 days. Two replicates of TN-1 and IET 1444 were prepared to determine repeatibility. After removal of the rice seedlings, the agar was adjusted to pH 3.0 using 0.06 M HCl to ensure that the exuded compounds were aglyconic and would match reference compound spectra. The samples were sonicated for 15 min.

Agar exudate samples were extracted with 3 $\times$ 60 ml aliquots of diethyl ether and the pooled ether layers were rotary evaporated until approximately 2 ml remained. After transferring the sample and ether rinsings to a minivial, the solution was concentrated further by nitrogen gas blow-down. Internal standard *p*-chlorobenzoic acid (0.5 ml of 10 μg/ml MeOH) was then added and the solution was evaporated to dryness using nitrogen. Samples were oven dried for 30 min at 60°C, followed by a further 30-min heating period after the addition of 1.0 ml of the silylating agent, bis(trimethylsilyl)trifluoroacetamide (BSTFA).

Identification Using Mass Spectral Libraries. The rT and mass spectral data contained in the MS and MS/MS user reference libraries were used in the identification of GC peaks obtained from rice root exudates. Positive identifications were made if chromatographic peaks in the samples had a spectral fit value of at least 700 (1000 is a perfect fit), and their rT were the same.

Quantitation of Exudate Analytes. Silylated root exudates from nine rice varieties, four allelopathic and five non-allelopathic, were prepared as previously described. The internal standard *p*-chlorobenzoic acid (*p*CBA) was included at a concentration of 5 μg/ml. Using the MS/MS method file previously developed, 1 μl of prepared exudate sample was injected into the GC in the splitless mode. Duplicate injections of an allelopathic cultivar, Takanenishiki, were made to check the precision of analyte/internal standard peak-count ratios. Because ratio variability was low, with less than 10% difference between chromatographic runs for each of the 15 quantified compounds, only one injection was made per prepared sample.

Calibration curves were initially constructed for all 20 compounds (Table 1). In the splitless mode, triplicate injections of seven calibration concentrations between 0.005 and 5 ppm were made using the first MS/MS method file already described. For each compound, a characteristic product ion was chosen from its MS/MS as the quantitation ion (Table 1). Calibration graphs for each separate analyte consisted of the ratio of chromatograph peak area of analyte to chromatographic peak area of internal standard (*p*CBA) versus analyte concentration (μg analyte/ml GC injection solution). Quantitation of 15 selected compounds was possible via these prepared calibration curves. The remaining five compounds

were either not present in the rice exudates, or reliable calibration curves could not be established under the experimental conditions.

Statistics. As raw data were skewed, the concentration results were natural log transformed and subjected to one-way ANOVA using Genstat 5, Release 3.2. The three most allelopathic and three least allelopathic cultivars as determined in previous screening experiments (Seal, 2003), were analyzed via least significant differences (l.s.d.).

RESULTS

GC/MS and GC/MS/MS Library Construction. Both the characteristic precursor ions selected from the GC/MS runs and the unique chromatographic retention times for 32 target compounds and the internal standard are displayed in Tables 1 and 2. Isolation of the characteristic ions of selected compounds followed by their collisional dissociation with helium atoms produced the quantifying daughter ions. One compound originally selected for analysis, ursolic acid, could not be characterized by GC/MS and was subsequently excluded from further analysis.

Identification of Compounds in Rice Root Exudates. Before adjusting the pH of the agar medium to 3.0 during sample preparation, both allelopathic and non-allelopathic cultivars had similar pH values. Therefore, any differences observed are unlikely to be due to pH effects. Through comparisons with our MS/MS user-library spectra of pure reference compounds and with standard retention times, 17 compounds were identified as present in the rice root exudates of both allelopathic and nonallelopathic varieties (Table 3). Although the spectral threshold level for positive identification was 700, in addition to matching retention times, the majority of compounds had a match factor of at least 800. Phenolics, phenylalkanoic acids, and indoles were among the chemical classes identified in the exudates. Gallic acid, α-hydroxybenzenepropanoic acid, and β-resorcylic acid were not detected in the root exudate samples.

Of the 11 compounds included in the qualitative second method file, 5 were detected in the rice root exudates of all allelopathic (Hungarian #1, IET 1444, and Giza 176) and nonallelopathic varieties (Woo Co Chin Yu and Basmati). 4-Vinylphenol, 3-hydroxybenzoic acid, α-resorcylic acid, hydrocaffeic acid, and 5-hydroxy-2-indolecarboxylic acid were identified in all five cultivars. Umbelliferone and 3-(4-hydroxyphenyl)propionic acid were also detected, in Basmati and Woo Co Chin Yu, respectively. The possible phytotoxins penicillamine, esculetin, scopoletin, and *o*-coumaric acid were not detected in any of the exudates.

Quantitation of Exudate Analytes. The regression coefficients (r) for the fitted linear regression equations of each calibration curve constructed from pure

TABLE 3. DERIVATIZED COMPOUNDS IDENTIFIED BY GC/MS/MS AND RT IN THE ETHER FRACTION FROM ROOT EXUDATES OF ALLELOPATHIC (A) AND NON-ALLELOPATHIC (NA) RICE CULTIVARS

TMS derivative of compound	rT	Class of compound	Number of cultivars[a]	
			A	NA
Mercaptoacetic acid	6.69	Thiol acid	3	3
Resorcinol	8.49	Phenol	4	5
Salicylic acid	10.28	Phenolic acid (benzoic)	4	5
4-Phenylbutyric acid	10.58	Phenylalkanoic acid	2	4
t-Cinnamic acid	10.93	Phenolic acid (cinnamic)	5	5
2-Hydroxyphenylacetic acid	11.16	Phenolic acid	3	5
α-Hydroxybenzenepropanoic acid	11.43	Phenylalkanoic acid	nd[b]	nd
p-Hydroxybenzoic acid	12.16	Phenolic acid (benzoic)	5	5
4-Hydroxyphenylacetic acid	12.36	Phenolic acid	4	4
Vanillic acid	14.51	Phenolic acid (benzoic)	5	5
β-Resorcylic acid	15.38	Phenolic acid (benzoic)	nd	nd
Indole-3-carboxaldehyde	16.25	Indole	5	5
Syringic acid	16.93	Phenolic acid (benzoic)	5	5
p-Coumaric acid	17.71	Phenolic acid (cinnamic)	5	5
Gallic acid	18.01	Phenolic acid (benzoic)	nd	nd
Indole-5-carboxylic acid	19.98	Indole	3	5
t-Ferulic acid	20.51	Phenolic acid (cinnamic)	5	4
Caffeic acid	21.33	Phenolic acid (cinnamic)	5	5
5-Hydroxyindole-3-acetic acid	22.68	Indole	5	4
Abietic acid	25.33	Terpenic acid	5	5

[a] Total number of cultivars examined (A + NA) was nine.
[b] nd represents compounds not detected with the GC/MS/MS file.

reference compounds are listed in Table 4. Fifteen of the seventeen compounds detected in the root exudates were quantified using GC/MS/MS analysis. As mercaptoacetic acid and indole-3-carboxaldehyde gave unreliable calibration curves they were excluded from quantitative analysis. Tables 5 and 6 list the concentrations of target analytes detected in the prepared root exudate samples. Reported errors represent standard errors of the estimation from the individual calibration curves. Statistical analysis of the mean concentrations determined from the allelopathic cultivar group (Table 5) and the non-allelopathic cultivar group (Table 6) indicates that significant differences between the two groups do exist. Of the 15 quantified compounds, 4 were significantly different, namely abietic acid, caffeic acid, *trans*-ferulic acid, and *p*-hydroxybenzoic acid (Figure 1). Phenolics compounds occurred in the highest amounts among the selected analytes quantified in the exudates. Of the seven highest ranked compounds, six were phenolic acids. Significant differences exist between the allelopathic and non-allelopathic cultivars in their production of three of these six compounds, caffeic acid, *trans*-ferulic

TABLE 4. LINEAR REGRESSION COEFFICIENTS FOR ROOT EXUDATE CALIBRATION CURVES

TMS derivative of compound	r^a	m^b	b^c
Resorcinol	0.984	0.05656	0.00447
Salicylic acid	0.983	0.00906	0.00049
4-Phenylbutyric acid	0.999	0.03256	0.00049
t-Cinnamic acid	1.000	0.02651	0.00056
2-Hydroxyphenylacetic acid	0.978	0.03862	0.00847
DL-β-Phenyllactic acid	0.998	0.03254	0.00064
p-Hydroxybenzoic acid	0.999	0.13163	0.00347
4-Hydroxyphenylacetic acid	0.998	0.11586	0.00385
Vanillic acid	0.999	0.20561	0.00278
Syringic acid	1.000	0.03513	0.00008
p-Coumaric acid	0.995	0.03543	−0.00083
Gallic acid	0.959	0.03585	−0.00404
Indole-5-carboxylic acid	0.997	0.03086	−0.00073
t-Ferulic acid	0.993	0.01448	−0.00275
Caffeic acid	0.998	0.07172	−0.00528
5-Hydroxyindole-3-acetic acid	0.992	0.04774	−0.00874
Abietic acid	1.000	0.05352	−0.00024

[a] linear regression coefficient for $Y = mX + b$ where Y is the peak area ratio of analyte to internal standard and X is the concentration of injected sample (μg/ml).
[b] Slope.
[c] Y – intercept.

acid, and *p*-hydroxybenzoic acid. No significant difference exists between the determined amount of total cinnamic acids and the total benzoic acids. The amount of terpenic compound, abietic acid, was significantly higher in the nonallelopathic cultivars.

DISCUSSION

Identification and quantitation of the compounds present in root exudates will provide useful information for researchers working towards the development of crop cultivars with allelopathic potential. Although tissue studies provide some information, exudate studies are more relevant to the source of compounds that exert effects on neighboring plants. In a study on wheat root, shoot, and exudates samples, Wu et al. (2001) found that the amount of phenolics quantified in the exudates was poorly correlated with the amount detected in roots and shoots, thereby stressing the importance of exudate studies.

In previous investigations on rice, indoles have been detected in tissue samples (Rimando et al., 2001). In addition to indole-5-carboxylic acid and indole-3-carboxaldehyde identified by Rimando et al. (2001), two previously unreported indoles have been detected in the present exudate study:

TABLE 5. CONCENTRATIONS OF TARGET ANALYTES DETERMINED BY GC/MS/MS ANALYSIS WITHIN ALLELOPATHIC RICE CULTIVAR EXUDATES

Common name	Calculated amount of chemical found (μg/l agar) allelopathic cultivars[a]			
	Giza 176	Hungarian #1	IET 1444	Takanenishiki
Resorcinol	0.03 ± 0.2[b]	0.03 ± 0.2	0.03 ± 0.2	0.03 ± 0.2
Salicylic acid	0.43 ± 0.017	0.30 ± 0.017	0.17 ± 0.017	0.20 ± 0.017
4-Phenylbutyric acid	0.03 ± 0.02	0.03 ± 0.02	nd[c]	nd
t-Cinnamic acid	0.2 ± 0.05	0.8 ± 0.05	nd	0.2 ± 0.05
2-Hydroxyphenylacetic acid	0.40 ± 0.48	0.033 ± 0.48	0.067 ± 0.48	0.13 ± 0.48
p-Hydroxybenzoic acid	4.37 ± 0.070	0.533 ± 0.070	1.17 ± 0.070	3.47 ± 0.070
4-Hydroxyphenylacetic acid	1.10 ± 0.082	0.267 ± 0.081	nd	0.23 ± 0.082
Vanillic acid	4.40 ± 0.130	0.333 ± 0.130	0.833 ± 0.130	1.07 ± 0.130
Syringic acid	1.63 ± 0.022	0.233 ± 0.022	0.300 ± 0.022	0.433 ± 0.022
p-Coumaric acid	1.20 ± 0.060	0.633 ± 0.060	0.733 ± 0.060	1.27 ± 0.060
Indole-5-carboxylic acid	0.2 ± 0.03	0.03 ± 0.03	0.03 ± 0.03	0.07 ± 0.03
t-Ferulic acid	5.50 ± 0.036	0.333 ± 0.036	3.00 ± 0.036	2.37 ± 0.036
Caffeic acid	1.80 ± 0.40	0.57 ± 0.40	0.67 ± 0.40	0.57 ± 0.40
5-Hydroxyindole-3-acetic acid	0.90 ± 0.16	1.03 ± 0.16	0.23 ± 0.16	0.33 ± 0.16
Abietic acid	5.70 ± 0.093	1.03 ± 0.093	2.13 ± 0.093	0.133 ± 0.093

[a] Cultivars demonstrating relatively high allelopathic potential in the ECAM bioassay.
[b] ±Standard errors of estimation as calculated from calibration curves.
[c] nd represents compounds not detected in the analysis.

5-hydroxy-2-indolecarboxylic acid and 5-hydroxyindole-3-acetic acid. Although the investigation by Mattice et al. (1998) characterized reference 5-hydroxy-2-indolecarboxylic acid via GC/MS and included it in their study, there was no mention of detecting the compound in rice tissue or soil. Several other compounds identified in our study have not previously been reported in rice root exudates. Mercaptoacetic acid, 4-vinylphenol, and hydroxyphenylacetic acid were also detected in both the allelopathic and relatively non-allelopathic cultivars selected for analysis.

Adom and Liu (2002) found that rice has ~5.6 μmol of gallic acid equiv/g of grain, and in previous rice allelopathy research, gallic acid was proposed as a potential rice allelochemical (Kuwatsuka and Shindo, 1973; Chou et al., 1991). There are several possible reasons why gallic acid was not detected in the rice root exudates examined here. One reason concerns the possible instability of the silanized derivative under the reaction conditions used here, and a second is the possibility that the chemical may not normally be released from the plant but instead remains inside the vacuole and other plant tissues.

Although there are drawbacks in attempting to relate plant allelopathy to donor plant tissue content, such studies have been widespread in allelopathy research. Thus, using HPLC techniques, Kong et al. (2002) evaluated over 3000

TABLE 6. CONCENTRATIONS OF TARGET ANALYTES DETERMINED BY GC/MS/MS ANALYSIS WITHIN NON-ALLELOPATHIC RICE CULTIVAR EXUDATES

Common name	Calculated amount of chemical found (μg/l agar) nonallelopathic cultivars				
	Toro	Basmati	Pelde	TN-1[a]	Woo Co Chin Yu
Resorcinol	0.03 ± 0.2[b]	0.03 ± 0.2	0.07 ± 0.2	0.03 ± 0.2	0.03 ± 0.2
Salicylic acid	0.17 ± 0.017	0.23 ± 0.017	0.27 ± 0.017	0.27 ± 0.017	0.27 ± 0.017
4-Phenylbutyric acid	nd[c]	0.10 ± 0.02	0.13 ± 0.02	0.07 ± 0.02	0.07 ± 0.02
t-Cinnamic acid	0.50 ± 0.05	0.23 ± 0.05	0.37 ± 0.05	0.17 ± 0.05	0.47 ± 0.05
2-Hydroxyphenylacetic acid	0.033 ± 0.48	0.033 ± 0.48	0.067 ± 0.48	0.067 ± 0.48	0.10 ± 0.48
p-Hydroxybenzoic acid	2.23 ± 0.070	0.967 ± 0.070	0.500 ± 0.070	0.867 ± 0.070	0.233 ± 0.070
4-Hydroxyphenylacetic acid	0.13 ± 0.082	nd	0.80 ± 0.082	0.17 ± 0.082	0.40 ± 0.082
Vanillic acid	0.500 ± 0.130	1.20 ± 0.130	0.667 ± 0.130	0.533 ± 0.130	0.700 ± 0.130
Syringic acid	0.27 ± 0.022	0.30 ± 0.022	0.33 ± 0.022	0.20 ± 0.022	0.27 ± 0.022
p-Coumaric acid	0.40 ± 0.060	0.47 ± 0.060	0.63 ± 0.060	0.83 ± 0.060	0.40 ± 0.060
Indole-5-carboxylic acid	0.07 ± 0.03	0.07 ± 0.03	0.13 ± 0.03	0.13 ± 0.03	0.17 ± 0.03
t-Ferulic acid	0.233 ± 0.036	0.633 ± 0.036	nd	2.20 ± 0.036	1.07 ± 0.036
Caffeic acid	0.27 ± 0.40	0.27 ± 0.40	0.27 ± 0.40	0.30 ± 0.40	0.27 ± 0.40
5-Hydroxyindole-3-acetic acid	0.23 ± 0.16	0.17 ± 0.16	0.47 ± 0.16	0.17 ± 0.16	nd
Abietic acid	2.83 ± 0.093	6.43 ± 0.093	14.10 ± 0.093	2.50 ± 0.093	6.63 ± 0.093

[a] TN-1 is cultivar Taichung Native 1.
[b] ±Standard error of estimation calculated from calibration curves.
[c] nd represents compounds not detected in the analysis.

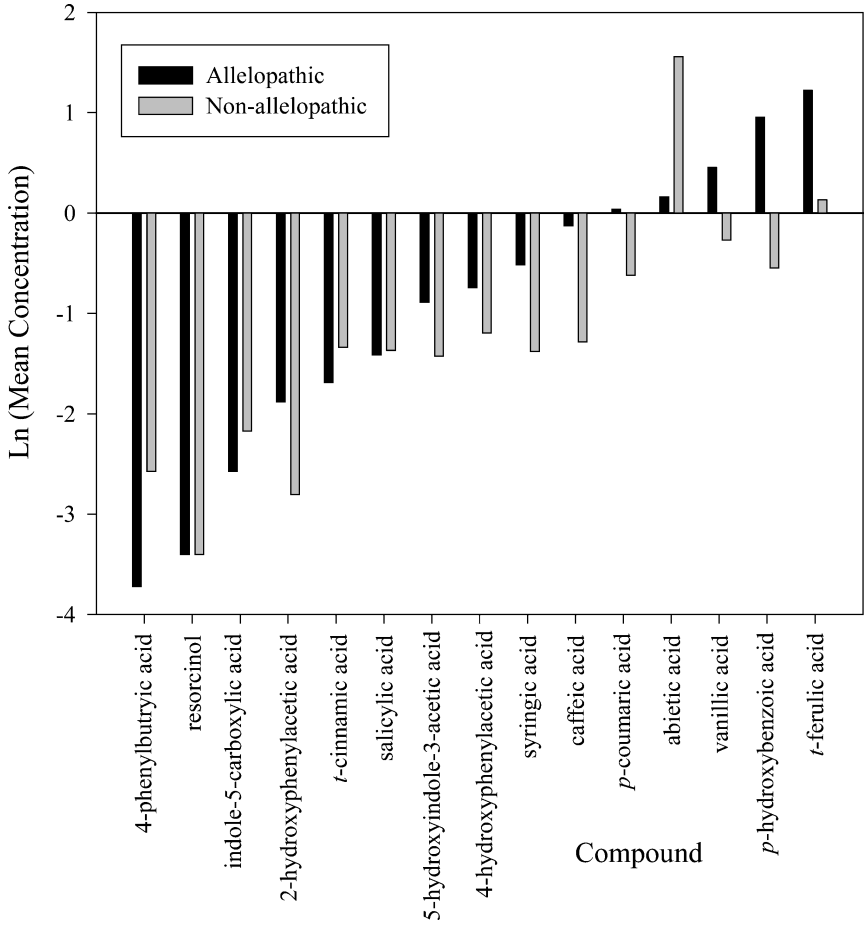

FIG. 1. Mean group concentrations of 15 target analytes detected by GC/MS/MS in allelopathic and nonallelopathic rice groups [l.s.d. (interactions) = 1.03, $P < 0.005$].

rice varieties for allelopathic potential. Through coupling of LC/MS and NMR, the potential allelochemicals in rice tissue were identified as glucosides of resorcinol, 2,4-dihydroxy-7-methylbenzoxazin-3-one (7-Me-DIBOA), and flavone. It is not unexpected that resorcinols are present in the rice exudate samples, as alkyl and alkenyl resorcinols are commonly found in cereal grains (Mullin and Emery, 1992). One such resorcinol, 5-(12-heptadecenyl)resorcinol, which is the main resorcinol found in rice root exudates, also serves as an antifungal agent in mango peel (Bouillant et al., 1994). Although resorcinol was detected in trace amounts in the

exudate, neither 7-Me-DIBOA nor flavone were detected. Once again, it could be a case of simply not being released from the plant.

Benzoxazinoids such as BOA (benzoxazolin-2-one), DIMBOA, and DIBOA have been isolated from many graminaceous species such as wheat (Wu et al., 1999, 2001; Belz and Hurle, 2001), rye (*Secale cereale*) (Barnes and Putnam, 1987), quackgrass (Friebe et al., 1995), and rice (Kong et al., 2002). In the present study, these compounds were not identified in the rice root exudates. Kong et al. (2002) has identified 7-Me-DIBOA in 7 of 3000 rice tissue samples using liquid chromatography/mass spectrometry (LC/MS). Selected ion searches on the ion chromatograms of root exudate samples from 10 varieties did not detect the presence of 7-Me-DIBOA, nor of 2 other prospective allelochemicals, asparagusic acid and 4-hydroxyhydrocinnamic acid. In addition, none of the indoles (including serotonin and gramine) could be detected using the same technique with simple GC/MS spectra. This reflects the complexity of the analytical samples and of the need for the greater selectivity of the GC/MS/MS technique (Van Pelt et al., 1998) used to quantify a selected range of target compounds for which we possessed authentic reference compounds.

One notable class of compounds identified in the exudates was the phenolics. The presence of phenolic acids in rice root exudates is not surprising as phenolic acids are ubiquitous within the plant kingdom. Friebe et al. (1995) found that vanillic acid, ferulic acid, and β-hydroxybutryic acid are allelochemicals in rye are also released by living quackgrass. Vanillic, *p*-hydroxybenzoic, ferulic, and syringic acids were detected in the plant parts of *Sorghum bicolor* (Ben-Hammouda et al., 1995). The exudates and tissues of alfalfa (*Medicago sativa*) contain caffeic, chlorogenic, isochlorogenic, *p*-coumaric, and *p*-hydroxybenzoic acid (Abdul-Raman and Habib, 1989). Kuwatsuka and Shindo (1973) have determined 13 phenolic acids in the ether fraction of the alkaline extracts of rice straw, cv. Kinmaze. Of these, *p*-coumaric, ferulic, vanillic, and *p*-hydroxybenzoic acids were present in the highest amounts. These same four compounds were most prominent in the rice root exudates examined in this study (Figure 1).

There were significant differences between the levels of several compounds released in the allelopathic rice varieties and those in the nonallelopathic varieties (Figure 1). It is not unprecedented that different cultivars can produce varying amounts of toxic compounds (Guenzi and McCalla, 1966; Wu et al., 2001). Lovett and Hoult (1992) found that the amount of phytotoxic alkaloids, hordenine, and gramine, varied in the tissue of 43 *Hordeum* cultivars, while Fay and Duke (1977) discussed the chemical variability of scopoletin production in 3000 oat cultivars. In this study, of the seven compounds in greatest amounts in the exudates, six were phenolic acids. Significant differences exist between the allelopathic and nonallelopathic cultivars in their production of three of these six compounds. Greater amounts of *trans*-ferulic acid, *p*-hydroxybenzoic acid, and

caffeic acid were detected in the exudates of allelopathic cultivars. The seventh compound, abietic acid, drawn to our attention by literature speculation on its possible micelle transportation capacity, was significantly higher in the nonallelopathic cultivars. Perhaps this compound has a "buffering" role in the sense that the presence of a larger amount of abietic acid in the root exudate counteracts the typical negative effects of potential allelochemicals on germination and plant growth..

The compared *trans*-ferulic acid was the most prominent phenolic detected in the allelopathic rice root exudates. This coincides with findings by Adom and Liu (2002), who reported that ferulic acid is the major phenolic found in cereal grains. Their study looked at free and soluble forms in addition to bound phenolics, which are often overlooked. Including bound phenolics in quantitation will provide a more accurate representation of the amounts present in cereal grain. According to Adom and Liu (2002), most phenolics in corn, wheat, oats and rice are found in the bound forms: 62% in rice. Bound phenolics are often associated with antioxidant activity (Adom and Liu, 2002). As such, it is possible that phenolic concentration influences availability of some elements in soil. Since antioxidants such as phenolics are preferentially oxidized, elements such as iron available to plants (Fe^{2+}) will not be oxidized to Fe^{3+}, an insoluble form that is unavailable to plants. Those plants with larger phenolic concentrations will be able to incorporate the iron even under oxidizing conditions, to the disadvantage of other plants growing nearby that do not have the same capability. This may increase the competitive ability of the crop via increased resource capture. Graminaceous plants sequester Fe from the rhizosphere differently from other monocots and dicots (Romheld, 1987). Phytosiderophores, which mobilize Fe, Zn, Mn, and Cu, are released by roots under suboptimal nutrient stress (Romheld, 1991). According to Romheld (1987), crops such as sorghum and rice can only acquire 20% of their Fe requirement through this mechanism, whereas barley can fully satisfy its Fe demands. Hence, Fe^{3+} reduction is crucial in determining nutrient status (Romheld, 1991). As iron oxides catalyze of phenolic acids (Huang et al., 1999), the level of iron oxides is reported to influence the speed of anaerobic decomposition of organic matter that is a nutrient source for rice (Tsutsuki and Ponnamperuma, 1986).

Generally, the amounts of individual compounds quantified from prepared rice exudates were small, especially when the required threshold concentrations for bioactivity are considered. Under our experimental conditions, there were no sources of stress which can trigger release of allelochemicals under typical field conditions (Rice, 1984).

Detection of these compounds in exudates does not imply that they actually are the causative allelochemicals. Determination of which compounds are responsible for the observed allelopathic effects of rice requires much further research, and is occupying our present investigations.

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EVALUATION OF PUTATIVE ALLELOCHEMICALS IN RICE ROOT EXUDATES FOR THEIR ROLE IN THE SUPPRESSION OF ARROWHEAD ROOT GROWTH

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Abstract—In previous studies, 15 putative allelopathic compounds detected in rice root exudates were quantified by GC/MS/MS. In this study, multiple regression analysis on these compounds determined that five selected phenolics, namely caffeic, *p*-hydroxybenzoic, vanillic, syringic, and *p*-coumaric acids, from rice exudates were best correlated with the observed allelopathic effect on arrowhead (*Sagittaria montevidensis*) root growth. Despite this positive association, determination of the phenolic acid dose-response curve established that the amount quantified in the exudates was much lower than the required threshold concentration for arrowhead inhibition. A similar dose-response curve resulted from a combination of all 15 quantified compounds. Significant differences between the amounts of *trans*-ferulic acid, abietic acid, and an indole also existed between allelopathic and non-allelopathic rice cultivars. The potential roles of these three compounds in rice allelopathy were examined by chemoassay. Overall, neither the addition of *trans*-ferulic acid nor 5-hydroxyindole-3-acetic acid to the phenolic mix significantly contributed to phytotoxicity, although at higher concentrations, *trans*-ferulic acid appeared to act antagonistically to the phytotoxic effects of the phenolic mix. The addition of abietic acid also decreased the inhibitory effect of the phenolic mix. These studies indicate that the compounds quantified are not directly responsible for the observed allelopathic response. It is possible that the amount of phenolic acids may be indirectly related to the chemicals finally responsible for the observed allelopathic effect.

Key Words—Abietic acid, decarboxylation, dose-response curves, GC/MS/MS, hydroxystyrenes, phenolic acids, rice allelopathy, *Sagittaria montevidensis*, tandem mass spectrometry, weed suppression.

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INTRODUCTION

The potential role of allelopathy in weed control has been the focus of much research and has been extensively reviewed (Einhellig and Leather, 1988; Purvis, 1990; Wu et al., 1999). Research groups worldwide are attempting to piece together the rice allelochemical puzzle. There are differing theories regarding the chemicals responsible for the allelopathic effect. Although much research has been done on the role of phenolics (Putnam, 1985), their importance in observed allelopathic effects remains a controversial issue. While Rimando et al. (2001) have identified phenolic acids present in rice from studies of allelopathic germplasm, Tanaka et al. (1990) doubt the importance of phenolic acids in the observed allelopathic effect, as the levels found in rice soils are not sufficient to produce phytotoxic effects. According to Olofsdotter et al. (2002), plant phenolics are usually present in flooded rice soils at concentrations below 5 mg/kg soil, which is below the bioactive threshold. Others argue that soil is nonuniform, and localized pockets of increased compound concentrations could build up in the rhizosphere (Dalton, 1989; Foy, 1999).

In addition to debate regarding their role in rice allelopathy, the possible synergistic action of phenolic acids also remains unclear. Some evidence suggests that phenolic acids are not synergistic, but rather antagonistic in their actions (Duke et al., 1983). This contradicts earlier published views about phenolic acids (Rasmussen and Einhellig, 1977, 1979). It appears that previous researchers may have drawn erroneous conclusions from incomplete data or have misread their dose-response curves (Duke, 2002). Duke (2002) stressed the importance of selecting an appropriate response parameter, the effect of physiological status on the response, and the importance of time courses in dose-response curves. Misread data could be responsible for the belief that phenolic acids occur in high enough concentrations to be responsible for observed allelopathic effects. Research by Belz et al. (2002) suggests that complete dose-response curves can be used to screen for allelopathic potential in target plant species.

The presence of chemicals in the root exudate does not infer that they play any role in the observed phytotoxicity. A typical chromatogram of rice root exudates contains around 200 peaks, such peaks potentially denoting at least 200 different compounds. The real challenge is to determine which compounds play a key role in the observed allelopathic effect. It has been postulated that allelopathic effects are more likely due to the combination and interaction of a complex mixture of compounds (Rizvi and Rizvi, 1992). This study focuses on the potential contributions of previously quantified rice exudate compounds (Seal et al., 2004) to the observed suppression of root growth of the rice aquatic weed arrowhead (*S. montevidensis*).

METHODS AND MATERIALS

Correlation Analysis. Multiple regression analyses (Microsoft Excel) were conducted to determine the correlation between various combinations of 15 previously quantified compounds (independent variables) from rice exudates and the corresponding level of inhibition of arrowhead root growth (dependent variable) across a range of rice cultivars. The root length values were natural log transformed prior to regression analysis. As there were nine cultivars and therefore eight degrees of freedom, up to seven independent variables could be examined. Hence, all 15 compound concentrations could not be individually applied in the same regression. Only when the compounds were grouped into classes such as cinnamic phenolics, benzoic phenolics, and indoles, could all 15 be analyzed together.

Preparation of the Pure Phenolic Mix. From the earlier quantitation work on rice root exudates (Seal *et al*., 2004a), five phenolics, identified as key components distinguishing allelopathic potential between rice varieties, were selected for the chemical bioassay. The phenolics were caffeic acid, *p*-hydroxybenzoic acid, vanillic acid, syringic acid, and *p*-coumaric acid. Using the molar phenolic ratio observed in the quantitation data (1.00:3.16:2.62:0.82:0.73), 0.0100, 0.0316, 0.0262, 0.0082, 0.0073, respectively, a stock solution of all five phenolics at 5 mM total was prepared in methanol. This phenolic mix was diluted to concentrations of 2000, 1000, 500, 200, 100, 50, 20, 10, 5, 2, and 1 μM in HPLC-grade methanol. The term "phenolic mix" represents this mixture of five phenolic acids.

Preparation of Additional Standards. Stock concentration solutions of 1000 μM were prepared for pure abietic acid, an abietic acid/phenolic acid mix, a *trans*-ferulic-acid/phenolic acid mix, a 5-hydroxyindole-3-acetic acid/phenolic acid mix, and a mix of all 15 quantified exudate compounds. All mixtures were made up using the ratios quantified previously. Stock solutions were diluted to concentrations of 500, 200, 100, 50, 20, 10, 5, and 1 μM in HPLC-grade methanol.

Chemoassays Using Reference Compounds. One milliliter of each of the above solutions was added to 250 ml beakers lined with Whatman #1 filter paper at the base. For the control, 1 ml of pure methanol was added. After the methanol had completely evaporated, 10 ml of sterile double distilled water (ddH_2O) was added. Ten pregerminated arrowhead seeds were sown directly into the water and the beaker covered with parafilm. Four replicates of each treatment were arranged in a randomized complete block design in a Precision Model 818 Low Temperature Incubator set at 27°C/22°C with a 12 hr day/12 hr night cycle. Seven days later the arrowhead seedlings were removed from the system and both their root and shoot lengths were measured to the nearest 0.5 mm.

Statistical Analysis and ED_{50} Estimates. All dose-response curves were subjected to one-way ANOVA using Genstat 5, Release 3.2. Arrowhead root length was taken as a percentage of control and least significant differences (l.s.d.) were

obtained. The 50% control test-plant root length response (Y) in chemoassay usually occurred between doses (X) of 500–1000 μM, a region where the curve is approximately linear. Effective dose (ED_{50}) values were estimated by determining the slope of the line between 500 and 1000 μM for each dose-response curve where $Y = mX + b$. Slope is represented by "m" and "b" represents the Y intercept. Using this equation, a corresponding value for dose (X) at the $Y = 50\%$ response figure could be calculated.

RESULTS

Correlation Analysis. Coefficients of determination for the 15 quantified individual compounds are listed in Table 1. Only one compound, 4-phenylbutryic acid, was significantly correlated with the inhibition of arrowhead root growth: a negative correlation, i.e., stimulation of growth, was observed ($r^2 = -0.49$). Greater levels of this compound were detected in the non-allelopathic cultivars than in the allelopathic ones. Other compounds such as resorcinol, indole-5-carboxylic acid, and abietic acid seemed also to be negatively associated with inhibition, although the correlations were not significant. While the total concentration of all

TABLE 1. COEFFICIENTS OF DETERMINATION BETWEEN ARROWHEAD ROOT LENGTH INHIBITION AND RICE EXUDATE COMPOUND LEVELS

Compound	Compound class	r^2 Value
Resorcinol	Phenol	−0.0041
Salicylic acid	Phenolic acid (b)[a]	0.0218
4-Phenylbutyric acid	Phenylalkanoic acid	−0.4910[b]
t-Cinnamic acid	Phenolic acid (c)	0.0029
2-Hydroxyphenylacetic acid	Phenolic acid	0.1906
p-Hydroxybenzoic acid	Phenolic acid (b)	0.2761
4-Hydroxyphenylacetic acid	Phenolic acid	0.0804
Vanillic acid	Phenolic acid (b)	0.0905
Syringic acid	Phenolic acid (b)	0.1587
p-Coumaric acid	Phenolic acid (c)	0.2675
Indole-5-carboxylic acid	Indole	−0.0102
t-Ferulic acid	Phenolic acid (c)	0.2444
Caffeic acid	Phenolic acid (c)	0.3387
5-Hydroxyindole-3-acetic acid	Indole	0.2645
Abietic acid	Terpenic acid	−0.1571
Total (all 15 compounds)	—	0.0282
Positive total	—	0.2574
Total (benzoics+cinnamics+indoles)	—	0.2577

[a] (b) represents benzoic acid type and (c) represents cinnamic acid type.
[b] Significant at $P < 0.05$.

TABLE 2. MULTIPLE REGRESSION ANALYSIS OF RICE EXUDATE COMPOUND LEVELS AND OBSERVED INHIBITION OF ARROWHEAD ROOT LENGTH

Compound	r^2 value
Benzoic acids[a]	0.7616
Cinnamic acids[b]	0.3768
Indoles[c]	0.3640
Benzoics+cinnamics+indoles	0.3952
Original phenolic mix[d]	0.9593[e]
Original phenolic mix + *t*-ferulic acid	0.9673
Original phenolic mix + 5-HI-3AA	0.9686

[a] Benzoics represents total benzoic acids.
[b] Cinnamics represents total cinnamic acids.
[c] Indoles represents total indoles.
[d] Phenolic mix consists of *p*-hydroxybenzoic, vanillic, syringic, caffeic, and *p*-coumaric acids.
[e] Significant at $P < 0.05$.

compounds quantified was not associated with inhibition ($r^2 = 0.03$), subtraction of negatively associated compounds increased the coefficient of determination to 0.26, though again such correlation was not significant.

Details on multiple regression analyses can be found in Table 2. Multiple regression analysis showed that the highest significant coefficient of determination ($r^2 = 0.96$) resulted from the regression of five selected phenolic acids: caffeic, syringic, *p*-coumaric, *p*-hydroxybenzoic, and vanillic acids. When examined individually, these compounds only accounted for 0.29–33.9% of the arrowhead root length variation and were not correlated with the inhibition of arrowhead root growth. Grouping of all the benzoic derivatives provided an r^2 value of 0.76, whereas the cinnamic acids produced an r^2 of 0.38. Neither group was associated with inhibition, nor was the level of indoles ($r^2 = 0.36$).

Chemical Bioassay Using Pure Phenolic Standards. The dose-response curve produced by the phenolic mix can be seen in Figure 1. Concentrations of 50 μM and below were not significantly different from the control (l.s.d. = 7.2, $P < 0.001$). All concentrations of phenolics above 1000 μM completely inhibited arrowhead root growth. Inhibition was below 30% in all concentrations of 200 μM and lower. The estimated ED_{50} for the pure phenolic curve was 502 μM.

Abietic Acid Dose–Response Curve. There were differences in the arrowhead root growth at different concentrations of abietic acid (Figure 1). All concentrations at or below 10 μM were not different from the control. Only the two highest concentration treatments (500 and 1000 μM) resulted in less root growth than the control. Concentrations between 10 and 500 μM increased arrowhead root growth (l.s.d. = 14, $P < 0.001$). Growth was most inhibited at the highest concentration

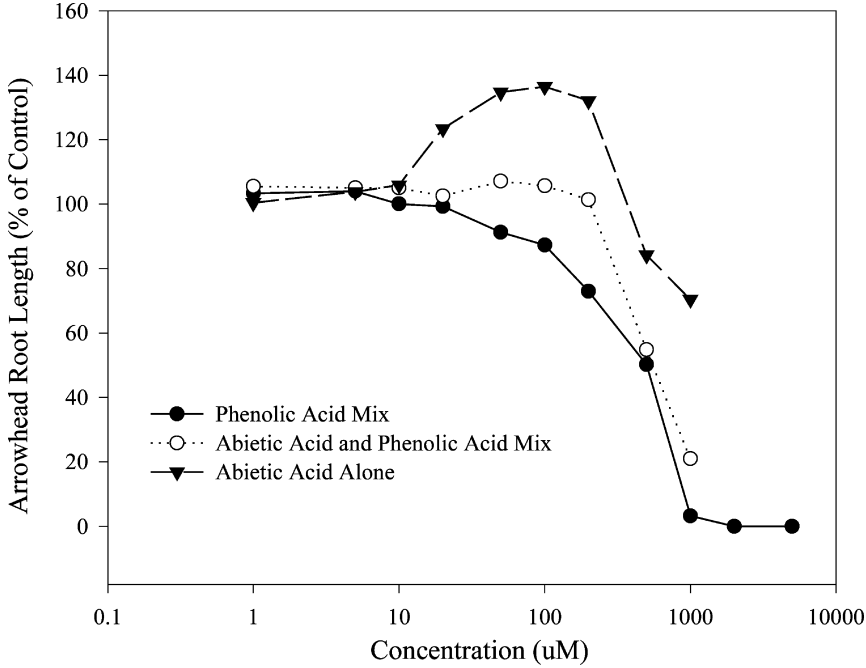

FIG. 1. Comparison of the abietic acid–phenolic mix curve with the phenolic acid and the abietic acid dose-response curves (l.s.d.$_{(\text{compound})} = 3.8$, l.s.d. (dose) $= 7.0$, l.s.d.$_{(\text{interaction})} = 12.1$, $P < 0.001$).

although root length was only reduced by 30%. When all tested concentrations are considered together, abietic acid actually stimulated arrowhead root growth, averaging 109% of the control. In this case, the highest concentration did not provide at least 50% reduction in arrowhead root growth, and therefore no estimation of ED_{50} was made.

Effect of Abietic Acid on the Phenolic Mix Dose–Response Curve. The dose-response curve for abietic acid and the phenolic acid mix (molar ratio 1.89:8.34) is shown in Figure 1. Only the two highest concentrations were significantly different from the control (l.s.d. $= 21.3$, $P < 0.001$). For the abietic acid/phenolic acid mix, the estimated ED_{50} was 571 μM. Overall, the addition of abietic acid to the phenolic mix affected the original dose-response curve of the phenolic mix. The main effect of concentration became significant at 200 μM and above in the abietic/phenolic mix and the phenolic mix alone. Interactions between compound mixes (chemical mixes refer to the specific chemical composition) and concentration also occurred. Below 50 μM, there were no interactive

effects. Arrowhead root length in all other concentrations except 500 μM was higher than in the original phenolics-only curve (l.s.d. = 11.6, $P < 0.005$). At 1000 μM, the highest concentration used in this experiment, the maximum inhibition was less than 80%, whereas with the pure phenolic mix, inhibition was greater than 99% (Figure 1). The addition of abietic acid resulted in a shift of the dose-response curve to the right, indicating an overall reduction in phytotoxicity.

Figure 1 shows all three abietic/phenolic dose-response curves. Significant differences do exist between all three curves (l.s.d.$_{(\text{compound})}$ = 3.8, $P < 0.001$). The main effects of concentration are significant (l.s.d. = 7.0, $P < 0.001$). When both concentration and compounds are considered, there are no differences in the responses at concentrations of 10 μM and lower (l.s.d.$_{(\text{interaction})}$ = 12.1, $P < 0.001$). At all higher concentrations except 500 μM, the arrowhead root length varied among all three chemical applications. At 500 μM, the inhibition due to abietic acid was less than in either the phenolic mix or the phenolic and abietic acid mix curves.

Effect of trans*-Ferulic Acid on the Phenolic Mix Dose–Response Curve.* Overall, the addition of *trans*-ferulic acid did not alter the original phenolic mix dose-response curve. The main effects of concentration became significant at and above 100 μM (l.s.d. = 9.7, $P < 0.001$) where the higher concentrations inhibited arrowhead root length (Figure 2). There were also interactions between the compound mixes and concentration (l.s.d. = 13.8, $P < 0.05$). In the 500 and 1000 μM treatments, the addition of *trans*-ferulic acid decreased inhibition. Instead of almost complete inhibition of arrowhead root growth at 1000 μM, only 80% suppression was observed. If an additive dose model is assumed here, then this result appears to support an antagonism between *trans*-ferulic acid and the other phenolic acids. However, nothing conclusive can be stated without a much more detailed statistical study based upon nonlinear models. The estimated ED_{50} for the mixture was 728 μM.

Effect of 5-Hydroxyindole-3-Acetic Acid on the Phenolic Mix Dose–Response Curve. The addition of 5-hydroxyindole-3-acetic acid to the phenolic mix decreased the inhibition of arrowhead root growth (l.s.d. = 3.7, $P < 0.02$). There were also differences due to concentration at the higher doses of 200, 500, and 1000 μM (l.s.d. = 8.3, $P < 0.001$). Below 200 μM, no differences in arrowhead root growth were observed (Figure 3). Despite these results, when both chemical composition and the concentrations were considered, there were no significant effects. The estimated ED_{50} for the phenolic–indole mix was 644 μM.

Chemoassay Using All Quantified Compounds. The dose-response curve for all 15 quantified potential allelochemicals can be seen in Figure 4. At 200 μM and below there are no effects of the compound mix on arrowhead root growth. At both 500 and 1000 μM, root growth was inhibited (l.s.d. = 13.9, $P < 0.001$).

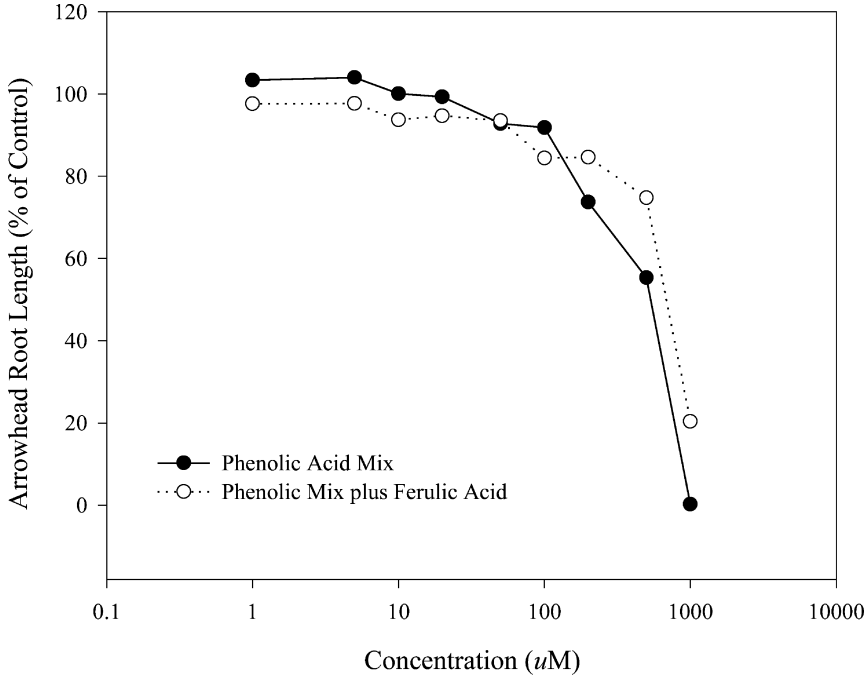

FIG. 2. The effect of *trans*-ferulic acid on the original phenolic mix dose-response curve (l.s.d.$_{(dose)} = 9.7$, $P < 0.001$, l.s.d.$_{(interactions)} = 13.8$, $P < 0.05$).

The allelochemical mix had an estimated ED_{50} of 569 μM. Table 3 summarizes the estimated ED_{50} values for all dose-response curves.

DISCUSSION

Multiple regression analysis determined five selected phenolics (the phenolic mix) to be best correlated with the observed allelopathic effect on arrowhead root growth. When examined individually, these five compounds had coefficients of determination ranging from 0.09 to 0.34. The pure compound bioassay revealed that the phenolic mix concentration required for 50% root length inhibition was 502 μM. The amount of this mix quantified from the average allelopathic group rice root exudate is much lower than the required threshold concentration indicated on its dose-response curve (Figure 1). At a total quantified concentration of <1 μM, the anticipated test-plant response actually lies in the slightly stimulatory section of the dose-response curve. This suggests that compounds other than just phenolics are responsible for the observed allelopathic effect, even though rice allelopathic

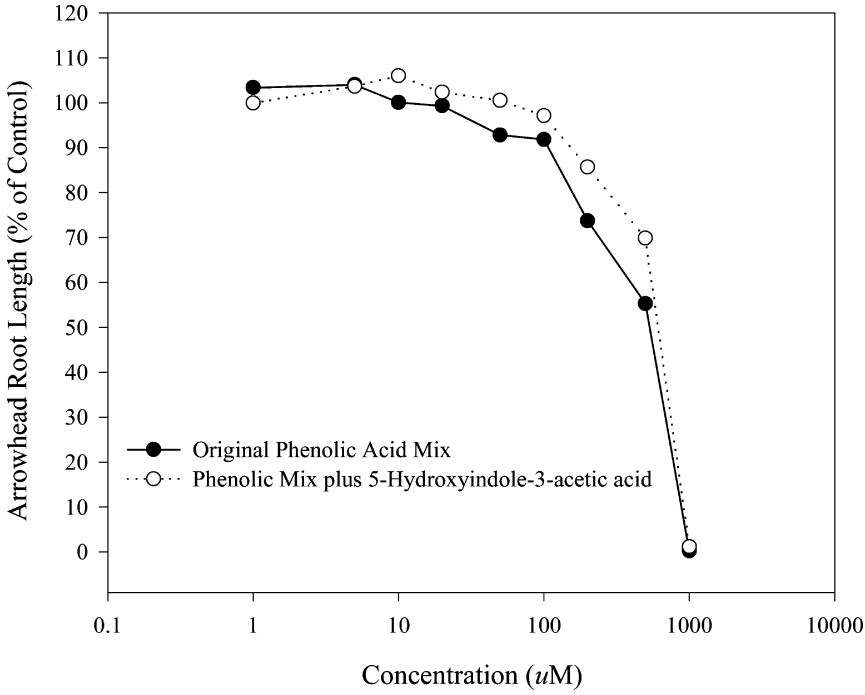

FIG. 3. The effect of 5-hydroxyindole-3-acetic acid on the original dose-response curve (l.s.d.$_{(\text{compound})} = 3.7$, $P < 0.02$, l.s.d.$_{(\text{dose})} = 8.3$, $P < 0.001$).

potential is positively associated with the concentration of these five key phenolics ($r^2 = 0.96$) (Table 2). The allelochemicals actually responsible may include toxic hydroxystyrenes that can be formed from relatively less toxic phenolic acids by decarboxylation (Liebl and Worsham, 1983). As such, the amount of phenolic acids may be indirectly related to the chemicals finally responsible for the observed allelopathic effect. Or it may simply be that the phenolic acids are playing a cell permeability role on the root cells of the receiver plant, enhancing uptake of more potent allelochemicals (Huang et al., 2003).

Research by Kobayashi et al. (1996) suggests that wheat plants, which belong to the same botanical family as rice, contain an enzyme in their roots which can decarboxylate cinnamic acids into their corresponding hydroxystyrenes (vinylphenols). Under sterile conditions, *p*-coumaric acid was added to hydroponic culture and was taken up by the wheat plant. Silica gel chromatography identified 4-hydroxystyrene (4-vinylphenol) from the culture medium. Similar results were obtained for ferulic acid with both 3-methoxy-4-hydroxystyrene and 3-methoxy-4-acetoxystyrene being identified. In an effort to determine whether

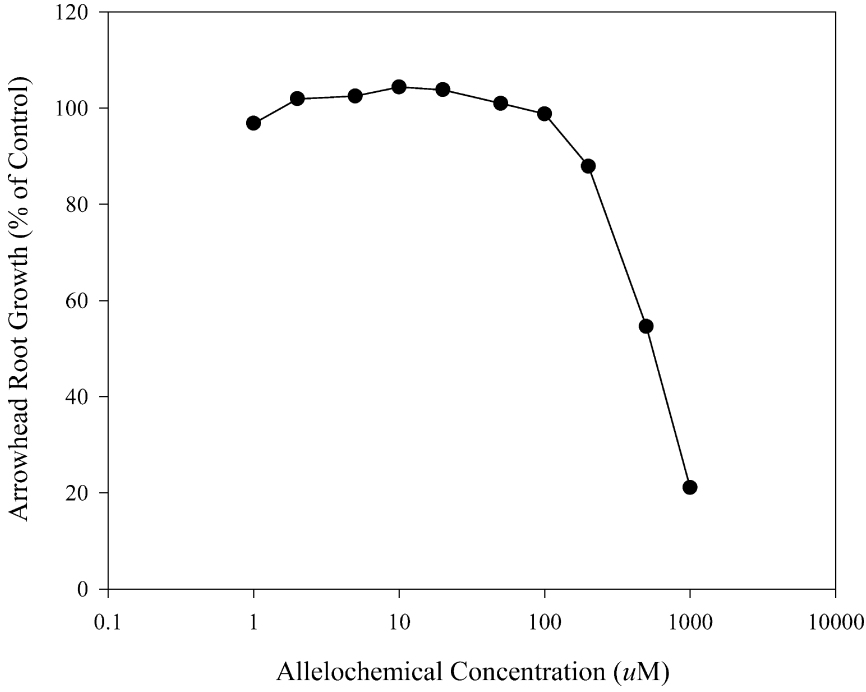

FIG. 4. Dose-response curve for mix of all 15 quantified compounds identified from the rice root exudates (l.s.d. = 13.9, $P < 0.001$).

these phenolic acids were converted into hydroxystyrenes by the wheat plant, ^{13}C-labeled *p*-coumaric acid was added to the culture medium. Detection of ^{13}C-labeled 4-vinylphenol provided evidence for this reaction. That this reaction occurs even under sterile conditions implies that the hydroxystyrenes are exuded by wheat plants after decarboxylation of phenolic acids inside the roots (Kobayashi et al., 1996). Further analysis by the Kobayashi group determined that the corresponding hydroxystyrenes have antimicrobial activity and some phytotoxicity. As 4-vinylphenol was detected in the rice root exudates of this study via high matches with library daughter mass spectra and retention time, it is possible that rice also possesses the mechanism whereby relatively nontoxic phenolic acids are converted to highly toxic hydroxystyrenes upon release into the environment. To examine this potential mechanism in rice, future research should include quantitation of identified hydroxystyrenes and determination of their role in the observed allelopathic activity.

Kong et al. (2002) also suggests that the responsible rice allelochemicals are not original exudates, but rather degradation products. Through coupling of

TABLE 3. DOSE OF COMPOUND MIXTURES REQUIRED TO SUPPRESS ARROWHEAD ROOT GROWTH BY 50%

Compound	Estimated ED_{50} (μM)
Phenolic acid mix (PAM)	502
Abietic acid and the PAM	571
trans-Ferulic acid and the PAM	728
5-Hydroxyindole-3-acetic acid and the PAM	644
All 15 quantified compounds	569

LC/MS and NMR, the potential allelochemicals in rice tissue were identified as glucosides of resorcinol, 7-methyl-DIBOA, and flavone. Kong et al. (2002) concluded that phenolics and fatty acids were not the allelochemicals of rice tissue, although these compounds could be the results of immediate breakdown of the glucosides of resorcinol, 7-methyl-DIBOA, and flavone after release into the environment.

Phenolics have been positively associated with allelopathic effects in a number of studies. Of eight compounds examined for allelopathic potential, only the concentrations of *p*-hydroxybenzoic, vanillic, and *trans*-ferulic acid quantified in water–agar were correlated with ryegrass root length (Wu et al., 2002). Individual concentrations of the eight compounds were not significant, whereas when considered together, there were significant associations (Wu et al., 2002). Joint interactions between compounds have also been reported by Chou et al. (1991) and Ben-Hammouda et al. (1995), although these studies focused on aqueous extracts of plant tissue. While such research is important in demonstrating the effects of compound mixtures, the concentrations quantified from root exudates would have more biological relevance. The static tissue concentration is not a good reflection of the levels of compounds that are actually released. Constant release over time may be required for concentrations to reach the threshold level (Dalton, 1989). According to Appel (1993), the expression of allelochemical effects is dependent on the threshold concentration.

This lack of proof of causation by phenolic acids, despite a good correlation with the observed allelopathic effects, stresses the importance of biological verification in allelopathic studies. The potential exists that relatively nontoxic compounds in ideal bioassay situations could play an important role in regulating plant germination and growth under stressful suboptimal environmental conditions (Weidenhamer and Romeo, 1989; Romeo and Weidenhamer, 1998). Seigler (1996) points out that bioassays measure the overall effects rather than the specific effects on plants, and therefore yield little information about allelopathic mechanism.

Although phenolic acid concentration and composition is not highly variable in this study, a differential allelopathic effect can still be realized in the field due to

differences in enzyme and oxidant activity (Appel, 1993). Appel (1993) stresses that oxidative conditions are an important determining factor in the actual effect of the phenolic acids. According to Ando et al. (1983), rice roots can oxidize compounds in the immediate rhizosphere presumably as defense against toxic compounds produced in submerged soils. This oxidizing power varies between rice cultivars (Armstrong, 1967). Those varieties typically grown in submerged soils may have become adapted to toxins by increasing oxygen release and/or oxidation power. Perhaps the expression of allelopathic effect depends not only on the concentration of phenolics released, but also on the ability of the cultivar to convert compounds into their bioactive form. This could account for some of the dissimilarity in comparable studies of rice allelopathy. One Egyptian cultivar, Giza 176, did not possess any allelopathic potential against barnyardgrass or dirty dora (Hassan et al., 1994) while ranking in the three most allelopathic rices against arrowhead (Seal et al., unpublished data). Perhaps differences in agronomic practice result in differential expression of allelopathic potential. Although similar amounts of allelochemicals can be present in exudates, oxidation ability is affected by nutrient status (Ando et al., 1983) and may not be sufficient under certain environmental conditions to convert the phenolic acids into the more toxic oxidized forms.

Among the 15 quantified rice exudate compounds examined in this study, 6 of the 7 highest concentrations in the exudates were phenolic acids. As discussed, 5 of these 6 were well correlated with the measured allelopathic effect on *S. montevidensis*. Significant differences between the amounts of the remaining phenolic (*trans*-ferulic acid) and abietic also existed between allelopathic and non-allelopathic cultivars. *trans*-Ferulic acid was greater in the allelopathic cultivars, whereas abietic acid was higher in the non-allelopathic cultivars. Because of these observed differences, the potential roles of these compounds in rice allelopathy were examined in more detail. Although 5-hydroxyindole-3-acetic acid was not significant, differences were considerable and as such, also examined in further detail.

When 5-hydroxyindole-3-acetic acid was added to the phenolic mix and tested for biological activity, it was found that this indole did not significantly affect the shape of the original dose-response curve (Figure 3), i.e., it did not contribute to the observed phytotoxicity. Similarly, *trans*-ferulic acid did not alter the phenolic mix dose-response curve overall, but at the higher concentrations of 500 and 1000 μM, *trans*-ferulic acid did reduce inhibition (Figure 2). It appears as though *trans*-ferulic acid acted antagonistically to the effects of the phenolic mix at higher concentrations, similar to findings by Duke et al. (1983).

Analysis of the effect of the terpenoid abietic acid on arrowhead plant growth showed that even at 1000 μM, a concentration unlikely to be found in nature, growth is only suppressed by 30% (Figure 1). Therefore, while this C_{20} compound is known for its micelle-forming ability, it is unlikely that abietic acid is

involved in the observed allelopathic effect on arrowhead root growth. Overall, abietic acid actually decreased the inhibitory effect of the phenolic mix (Figure 1). The possibility exists that abietic acid has a "buffering" role and counteracts the typical negative effects of allelopathy on germination and plant growth of the other compounds present in the rice root exudates. Perhaps it is the amount of abietic acid released by the cultivar that dictates its allelopathic potential. In general, the allelopathic potential of varieties examined in this study displayed a negative correlation with the amount of abietic acid. The greater the amount of abietic acid, the weaker was the observed allelopathic effect.

Besides abietic acid, the amounts of three other compounds resorcinol, 4-phenylbutyric acid, and indole-5-carboxylic acid showed a slight negative correlation with the inhibition of arrowhead root length. Of these, only 4-phenylbutyric acid was significantly correlated, with an r^2 value of -0.49 (Table 1). Resorcinol was the least variable of the 15 compounds quantified across both allelopathic and non-allelopathic cultivars. Only one of the nine rice varieties differed from the otherwise constant value of 0.0333 μg/l agar. Pelde, a weakly allelopathic variety, had twice this amount of resorcinol. Although resorcinol is a toxic compound, it does not appear to be involved in the differences in allelopathic potential observed in our biological assay.

The full range of 15 compounds was tested collectively for allelopathic potential against arrowhead in the natural ratios quantified by GC/MS/MS analysis. As with the phenolic acid mix, the levels required for inhibition far exceed the amount quantified in the rice root exudates. Correlation between total level of compounds and arrowhead suppression was very low at 0.03. When those individual compounds with negative r^2 values were removed, the coefficient improved, increasing to 0.26. As yet, all the compounds involved in rice allelopathy remain to be determined and characterized. It is possible that the amounts of compounds released by plants in the field are much higher than plants grown in the glasshouse or the laboratory. In a study by Hall et al. (1982), sunflower plants grown in the field contained 58% more phenolic compounds than plants grown in the greenhouse. Perhaps the ideal rice growing conditions of the incubator excluded any stresses that would promote compound exudation.

Most chemicals implicated in rice allelopathy have been found ubiquitously throughout the rice germplasm. This raises the question of selectivity. Why are the varieties that are more allelopathic toward barnyard grass not consistently allelopathic toward other weeds such as arrowhead or ducksalad, even though the compounds isolated from the varieties are similar? Besides the important dose or concentrations effects, factors influencing the selectivity of allelopathy could include the sensitivity of the species, the presence of endophytes, and soil characteristics. Of course, the alternative exists that the responsible compounds have not been isolated and the key compounds remain undiscovered. Worsham (1989) reported that less than 5% of the secondary metabolites from plants have

been isolated and identified, which leaves much scope for the possible involvement of other compounds.

Several putative allelochemicals appear to have defensive roles such as antimicrobial and antifungal functions, for example, hydroxystyrenes (Kobayashi et al., 1996) and phytoalexins (Cartwright et al., 1977, 1981). Perhaps the presence of protective allelochemicals in crops predisposes weeds growing in the vicinity to be preferentially subject to microbial attack, thereby resulting in reduced growth. The observed allelopathic effect could be the sum of direct chemical influence and/or indirect mechanisms such as increased susceptibility to microbial attack, thereby opening the possibility for an array of mechanisms of action.

Although this study did not determine the responsible allelochemical components of rice root exudates, a number of clues were uncovered. It is through such chemical analysis that the complexity of allelochemical interactions becomes apparent. While this study determined that phenolics are not directly responsible for the observed allelopathic effect, the findings do suggest that phenolics are either indirectly related to the responsible allelochemicals or play a joint role in the observed phytotoxicity. Also, none of the remaining identified compounds in the rice root exudates appears to be directly responsible for the observed allelopathic effect. It is possible that the key compounds remain undiscovered and/or the ideal compound ratio has yet to be established. Einhellig and Leather (1998) suggest that only a small number of secondary compounds have been examined for herbicidal activity. According to Einhellig (1996), it is possible that multiple compounds present at concentrations below the activity threshold can have pronounced allelopathic effects through their joint action, though evidence for this idea in practice is elusive. This study is one of the first to report the phytotoxic effects of chemical mixtures on root growth using the amounts quantified from living rice roots as a basis. Future research would benefit from a focus on identification of the complete array of chemical components in rice root exudates. In addition, determination of the combined effects of multiple chemicals as quantified in the exudates is imperative if the chemical basis of rice allelopathy is to be established.

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DO SALIVARY PROLINE-RICH PROTEINS COUNTERACT DIETARY HYDROLYZABLE TANNIN IN LABORATORY RATS?

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Abstract—We hypothesized that dietary hydrolyzable tannins would not act as digestibility reducing substances but would be excreted in the feces if the tannin were ingested by rats producing salivary proline-rich proteins (PRPs). To test that hypothesis we used two groups of Sprague–Dawley rats: tannin-naïve rats that were secreting basal levels of salivary PRPs and tannin-habituated rats that were secreting elevated levels of PRPs. The animals were fed for 10–18 d on diets containing 3% (w/w) purified hydrolyzable tannin [pentagalloyl glucose (PGG)] that was periodically spiked with chemically synthesized, radiolabeled 1,2,3,4,6-penta-*O*-galloyl-[U-^{14}C]-D-glucopyranose (1 μCi per gram diet). The PGG-habituated rats excreted three times more of the consumed ^{14}C in their feces than did the PGG-naïve rats (11.4% for PGG-habituated rats *vs*. 3.5% for PGG-naïve rats, $P < 0.05$). The addition of 3% PGG to the diet of the PGG-naïve rats had no significant effect on apparent dry matter or nitrogen digestibility ($P > 0.05$). However, dry matter digestibility and nitrogen digestibility were significantly decreased by PGG in the diets of the PGG-habituated rats (7 and 25%, $P < 0.001$, respectively). Production of PRPs increased the amount of PGG excreted intact in the feces but at the cost of diminishing apparent dry matter and nitrogen digestibility.

Key Words—Tannin, pentagalloyl glucose, proline-rich protein, apparent digestibility, laboratory rat.

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INTRODUCTION

Tannins are polyphenolic natural products that readily bind protein under conditions that are present in the gastrointestinal tract of many species (Hagerman et al., 1998). The presence of tannins in a foodstuff may reduce palatability, limit digestibility, and increase toxic load (Mole et al., 1990). There are two main subgroups of tannins, condensed and hydrolyzable tannins (Haslam, 1989). Condensed tannins are polymers of flavan-3-ols that are linked *via* interflavan bonds and are not readily susceptible to degradation. Hydrolyzable tannins have a polyol core (oftentimes glucose) esterifed to simple phenolic acids such as gallic acid. Hydrolyzable tannins are susceptible to acid, base, or enzyme-catalyzed hydrolysis, yielding small phenolic acids, such as gallic acid and glucose. Hydrolysis in the mammalian digestive tract (Murdiati et al., 1991; Bravo et al., 1994) releases phenolic acids that may be locally toxic to gut microflora (Lowry et al., 1996) or may be absorbed from the intestine and cause systemic toxicity within the animal (Niho et al., 2001). Even if the amount of phenolic acid absorbed from the intestine is below the toxic dose, a metabolic cost may be paid by the animal to detoxify and excrete the absorbed phenolics (Thomas et al., 1988; Illius and Jessop, 1995).

The fundamental characteristic of tannins is their propensity to precipitate proteins, and it has long been hypothesized that tannins reduce nitrogen digestibility by binding to dietary protein and digestive enzymes in the gut (Austin et al., 1989; Robbins et al., 1991; McArthur et al., 1995). This idea was challenged after it was found that tannic acid—a mixture of hydrolyzable tannins and low molecular weight phenolics (Hagerman et al., 1992)—reduces nutrient assimilation by a different mechanism than reducing digestibility (Bernays et al., 1989; Mole et al., 1990, 1993). However, because tannic acid is a mixture containing both tannins and low molecular weight phenolics (Hagerman et al., 1992), it is unclear whether the reductions in nutrient assimilation are due to tannins in the tannic acid or due to the toxicity of the low molecular weight phenolics.

When consuming tannins, some mammals secrete salivary proline-rich proteins (PRPs) that bind to the tannins with high affinity (Hagerman and Butler, 1981; Mehansho et al., 1983, 1992). It has been proposed that the salivary PRPs bind to the tannins in the gastrointestinal tract, forming indigestible complexes. The complexed tannins may escape both enzymatic and microbial degradation and be excreted in the feces, thus protecting the animal from both digestibility reduction and toxicity (Austin et al., 1989). However, the lack of a suitable analytical method to quantitate the amount of tannin in fecal samples has hindered tests of this hypothesis (Hagerman and Butler, 1989; Mole et al., 1993; Mueller-Harvey, 2001).

Laboratory rats normally secrete only low levels of salivary PRPs but tannin-containing diets induce production and secretion of salivary PRPs (Mehansho et al., 1983, 1992). Because PRP secretion is an inducible response in laboratory

rats, they make an ideal test species to determine the role of PRP secretion in counteracting dietary tannin because it is possible to evaluate the physiological effects of low *vs*. high PRP production in not only the same species, but in the same individuals. Also laboratory rats are reasonable models for the many species that share their digestive strategy of little pregastric fermentation, digestion of cell solubles primarily in the small intestine, followed by hindgut fermentation of some undigested residues. To take advantage of the inducibility of PRP secretion, we used two groups of rats: PGG-habituated rats, which ingested PGG for 8 d pretrial in order to induce PRP production, and PGG-naïve rats, which had never consumed PGG and were not secreting PRPs when the experiment started. We used a pure, homogeneous hydrolyzable tannin 1,2,3,4,6-penta-*O*-galloyl-D-glucopyranose [pentagalloyl glucose (PGG)], spiked with chemically synthesized, radiolabeled pentagalloyl glucose (^{14}C-PGG) to directly investigate the fate and consequences of ingested hydrolyzable tannins in laboratory rats.

METHODS AND MATERIALS

Animals and Housing. Ten 6-wk-old, male Sprague–Dawley rats (Harlan Teklad, Madison, WI) weighing 145–160 g were housed individually in plastic metabolic cages (Nalge Nunc International, Rochester, NY) that allowed complete separation of urine and feces. The rats were placed on a reverse light cycle of 12D:12L with an ambient temperature of 25°C and a relative humidity of 40%. All procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the Research Animal Resource Center Animal Care Committee at the University of Wisconsin, Madison.

Diet. AIN-76A powdered diet was used as the basal diet (see Table 1 for composition; American Institute of Nitrition, 1977). PGG, the test tannin, was

TABLE 1. COMPOSITION OF DIETS

Ingredients[a]	Basal diet (g/kg)	3% PGG diet (g/kg)
Casein	200	200
DL-Methionine	3	3
Sucrose	500	500
Corn starch	150	150
Corn Oil	50	50
Cellulose	50	20
Pentagalloyl glucose	0	30
Mineral Mix, AIN-76	35	35
Vitamin Mix, AIN-76A	10	10
Choline bitartrate	2	2

[a] All ingredients except pentagalloyl glucose were from Teklad Inc., Madison, WI.

added to the diet on a 3% per dry weight basis in place of cellulose (hereafter referred to as the 3% PGG diet). The PGG was added daily to the diet before feeding to minimize oxidation.

Pentagalloyl Glucose. PGG was purified from commercially available tannic acid (Sigma-Aldrich, St. Louis, MO) as described by Hagerman et al. (1997). The radiolabeled 1,2,3,4,6-penta-*O*-galloyl-[U-^{14}C]-D-glucopyranose was chemically synthesized from [U-^{14}C]-D-glucose (Perkin–Elmer Life Science, Boston, MA) and tri-*O*-benzylgallic acid (Sigma-Aldrich, St. Louis, MO) and had a radiopurity of at least 95% (Chen et al., 2003). The structure and purity of the natural and synthetic PGG were established by HPLC, MS, and NMR (Chen et al., 2003) before the feeding trials started and after the trials were completed to confirm compound integrity throughout the experiment. Both the unlabeled as well as radiolabeled PGG were stored at –80°C when not in use.

Experimental Procedure. Rats that had been consuming the basal diet for 10 d were randomly assigned to two groups of five rats each, the PGG-habituated or PGG-naïve group. For the first 8 d of the experiment, the five rats assigned to the PGG-habituated group consumed the 3% PGG diet, while the five rats assigned to the PGG-naïve group were maintained on the basal diet (Figure 1). Food intake and body weight were measured daily. Feces uncontaminated by urine were collected every 24 hr and frozen.

After 8 d of habituation, the experimental period was initiated. During the experimental period, all rats received the 3% PGG diet. Rats reduce their feed intake when they are initially introduced to a PGG-containing diet (Table 2), so in order to ensure an equivalent PGG dose between the two groups we utilized a pair-feeding design during the experimental period. Each rat in the PGG-habituated group was paired with a rat in the PGG-naïve group and was fed only the amount of 3% PGG diet consumed by the PGG-naïve rat with which it was paired. On d 1, 3, 5, and 7 of the experimental period, the rats were fed a 5-g meal of the 3% PGG diet with 1 μCi ^{14}C-PGG per gram of food. In a preliminary study, the maximum residence time of ^{14}C-PGG fed to either PGG-naïve or PGG-habituated rats was 48 hr, so the rats were dosed with the radiolabeled PGG every other day. Rats were fed the ^{14}C-PGG diet in shoebox cages instead of the metabolic

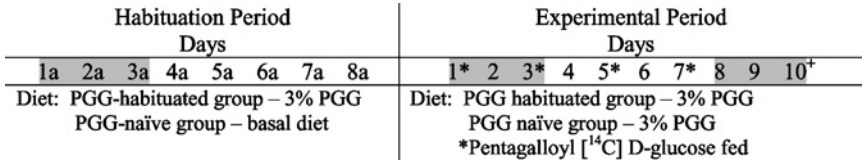

FIG. 1. Experimental timeline. Days highlighted in gray were used to determine the 3-d averages of food intake, weight gain, proline excretion, and apparent dry matter and nitrogen digestibility.

TABLE 2. FOOD INTAKE AND WEIGHT GAIN

Days	Group	Diet	Food intake[a] (g/d)	Weight gain[a] (g/d)
1a–3a	PGG-habituated	3% PGG	$7.6 \pm 0.6_{d}$	$-2.1 \pm 0.6_{c}$
1a–3a	PGG-naïve	Basal	$14.4 \pm 0.7_{a}$	$5.9 \pm 0.1_{a}$
1–3	PGG-habituated	3% PGG	$9.3 \pm 0.4_{c}$	$1.2 \pm 0.7_{b}$
1–3	PGG-naïve	3% PGG	$10.2 \pm 0.3_{c}$	$-0.1 \pm 0.2_{c}$
8–10	PGG-habituated	3% PGG	$12.9 \pm 0.2_{b}$	$2.8 \pm 1.6_{b}$
8–10	PGG-naive	3% PGG	$12.8 \pm 0.3_{b}$	$3.2 \pm 0.4_{b}$

[a] Data given are the mean ± SE of the 3-d averages for each group of five rats. Numbers within a column with different subscript letters are significantly different ($P < 0.05$) as determined by Tukey's HSD.

cages to prevent contamination of the 24-hr fecal collections with spilled food. The 5-g meal containing the ^{14}C-PGG was fed within 30 min of the start of the dark cycle, and rats were allowed to feed for 1 hr. Before the rats were placed back in their respective metabolic cages, they were thoroughly wiped with wet paper towels to remove any diet particles present on their fur, tails, or whiskers. The nonradiolabeled 3% PGG diet was then provided to the rats in the metabolic cages. Food intake and body weight were measured daily, and feces were collected as described above.

Fecal Analysis. Fecal samples were lyophilized and reweighed to determine dry matter content. Dried fecal samples were ground using a mortar and pestle and partitioned for liquid scintillation counting (LSC), proline analysis, and nitrogen analysis.

Radioactivity in the feces was used as a measure of the amount of ^{14}C-PGG that was excreted in the feces. The labeled PGG was synthesized using [^{14}C]-D-glucopyranose, and so hydrolysis of the ^{14}C-PGG in the stomach or small intestine of the rats would release [^{14}C]-D-glucose that would be readily absorbed and metabolized, not excreted in the feces. However, chemical degradation studies in artificial gastric or intestinal fluid demonstrate stability of PGG to hydrolysis for at least 24 hr (Hagerman and Carando, unpublished data). Similarly, Bravo et al. (1994) found that tannic acid is a poor substrate for fermentation by rat ceacal microflora, and that 75–95% of the initial tannic acid added was recovered after fermentation. Since PGG is one of the polyphenolic compounds present in tannic acid it was assumed that the ^{14}C-PGG that reached the rat's hindgut would not be readily fermented and, therefore, would be excreted intact in the feces. For LSC, 0.02 g of feces were rehydrated in 8-ml scintillation vials with 0.1 ml of deionized water. The rehydrated feces were solubilized by adding 1 ml of Soluene 350 (Sigma-Aldrich, St. Louis, MO) and placing the vials in a 50°C oven until all particulate matter had been dissolved. Five microliter of scintillation fluid (Ecolume, ICN Costa Mesa, CA) containing 5% acetic acid (to decrease chemiluminesence) were added to the sample vials, and the samples were counted

to obtain dpm ^{14}C with a Wallac WinSpectral 1414 liquid scintillation counter (Perkin–Elmer, Wellesley, MA).

Ground-dried feces were also analyzed for proline content to determine if the rats were excreting PRPs. Fecal proline excretion was used as an index for the induction and secretion of salivary PRPs (Jansman et al., 1994) in order to avoid the stress caused by saliva collection. Fecal samples were analyzed for proline using a 24-hr acid hydrolysis in 6 N HCl at 100°C under nitrogen followed by a colorimetric ninhydrin reaction (Magne and Larher, 1992). Total proline (free and protein) is detected by this method.

Ground-dried feces were also analyzed for nitrogen content to determine the effect of PRPs on apparent nitrogen digestibility. The nitrogen content of the feces was measured via a micro-Kjedhal digestion followed by a phenol-hypochlorite colorimetric determination of ammonia (Weatherburn, 1967).

Diet Analysis. The diets were analyzed for dry matter content by heating them in 50°C drying oven. The nitrogen and proline contents of the diet were analyzed as described for fecal samples. Subsamples of the ^{14}C-PGG diet fed each day were analyzed via LSC.

Dry Matter Calculations. Apparent dry matter (DM) digestibility was calculated on a 24 hr basis. DM digestibility = [(gram DM intake) – (gram dry weight of feces)]/[gram DM intake].

To calculate the amount of fecal DM excreted as PRPs it was assumed that rat PRPs are 40% proline (Mehansho et al., 1983). PRP excretion = (gram proline in feces)/0.40.

To calculate the mass of PGG in the feces of animals consuming 3% PGG in the diet it was assumed that ^{14}C fecal excretion was proportional to total PGG excretion. PGG excreted = [(gram food intake) × 0.03] × percent ^{14}C in feces.

Nitrogen Calculations. Apparent nitrogen (N) digestibility was calculated on a 24 hr basis. Apparent N digestibility = [(N intake) – (fecal N excretion)]/[N intake]

To calculate the amount of fecal nitrogen excreted as PRPs it was assumed that rat PRPs are 40% proline (Mehansho et al., 1983) and 16% nitrogen. N excreted as PRPs = [(gram proline in feces)/0.40] × 0.16.

Calculations of Reductions in Digestibility. Reductions in apparent N and DM digestibility were calculated as the difference in the 3-d average between d 1a–3a and 8–10. Reduction in digestibility = [(digestibility d 1a–3a) – (digestibility d 8–10)]/[digestibility d 1a–3a].

^{14}C-PGG Dose Calculation. The dosage of ^{14}C-PGG consumed was calculated based on the concentration of ^{14}C in the diet and food intake. ^{14}C-PGG dose = [dpm ^{14}C/gram diet] × [gram diet eaten].

Statistical Analysis. Food intake, weight gain, fecal proline, and apparent dry matter and nitrogen digestibility were analyzed as 3-d averages. Apparent

dry matter and nitrogen digestibility, % ^{14}C excreted in feces and % reductions in apparent dry matter and nitrogen digestibility were normalized by an arcsine square root transformation. Data were analyzed by repeated measure analysis of variance (ANOVA) with treatment and time as factors using SYSTAT (Wilkinson and Coward, 2000). Differences between individual means were determined by Tukey's honestly significant difference (HSD). All data are expressed as mean $\pm$ SE unless stated otherwise and P values of 0.05 or less were used to determine significance.

RESULTS

Food Intake. During the habituation period, food intake by the PGG-consuming group diminished to almost half that of the PGG-naïve group (compare between groups on d 1a–3a in Table 2, $P < 0.05$). During the experimental period, the rats in the PGG-habituated group were pair fed to rats in the PGG-naïve group to eliminate differences in food intake (compare between groups on d 1–3 and 8–10 in Table 2). As the rats became habituated to the 3% PGG diet, their food intake increased (compare within groups on d 1–3 and 8–10 in Table 2, $P < 0.05$).

Weight Gain. As expected based on their low food intake, the rats initially lost weight on the 3% PGG diet (Table 2, compare between groups on d 1a–3a and within the tannin naïve-group on d 1a–3a and 1–3, $P < 0.05$). As the rats became habituated to the dietary PGG, their weight gain increased but did not reach that of the animals not consuming PGG (Table 2, compare d 8–10 to PGG-naïve d 1a–3a, $P < 0.05$).

Proline Excretion. The total proline defecated (mg/d) increased as the rats became habituated to the 3% PGG diet (Figure 2) with maximal PRP excretion 8–10 d after initiating the tannin diet (Mehansho et al., 1983). Rats that had been on 3% PGG diets for 10–11 d had almost 10 times more proline in their feces than those on either the basal diet or on the 3% PGG diet for only 3 d (Figure 2, 42.2 mg/d, d 1–3 *vs.* 4.6 mg/d, d 1a–3a in the PGG-habituated group, $P < 0.05$, and 32.5 mg/d, d 8–10 *vs.* 4.1 mg/d, d 1a–3a in the PGG-naïve group, $P < 0.05$). The increase in fecal proline is coincident with the induction of salivary PRPs (Mehansho et al., 1983), suggesting that the proline is derived from PRPs. However, other endogenous proteins such as mucins and sloughed cells, or bacterial proteins, could also contribute to fecal protein and consequently fecal proline.

^{14}C in Feces. PGG habituation increased fecal excretion of ^{14}C over threefold (Figure 3, d 2, PGG-habituated excreted 11.4% of ingested ^{14}C *vs.* naïve excretion of 3.5%, $P < 0.05$). Fecal excretion of ^{14}C increased as the PGG-naïve group became habituated to PGG (Figure 3, d 2 *vs.* d 8, $P < 0.05$). The PGG-habituated group also excreted significantly more ^{14}C as the experiment progressed (Figure 3,

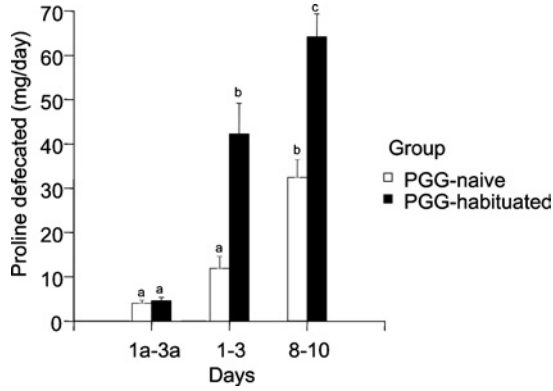

FIG. 2. Amount of proline defecated as rats habituated to PGG consumption. The PGG-habituated group was placed on the 3% PGG diet starting d 1a and had been eating the 3% PGG diet for 9 d by d 1. The PGG-naïve group was on the basal diet on d 1a–3a and was placed on the 3% PGG diet on d 1. Data are represented as the mean ±SE. Bars with different letters (a, b, c, d, and e) denote means significantly different from each other ($P < 0.05$) as determined by Tukey's HSD.

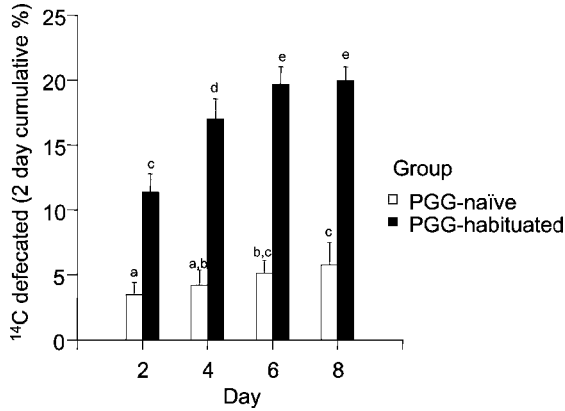

FIG. 3. Percent of ^{14}C consumed that was defecated within 48 hr in PGG-habituated rats *vs*. PGG-naïve rats. Rats were dosed with pentagalloyl [^{14}C]-D-glucose on d 1, 3, 5, and 7 and feces were analyzed on a 48 hr basis. The PGG-habituated group had been on the 3% PGG diet for 8 d prior to d 1 while the PGG-naïve group was first exposed to the 3% PGG diet on d 1. Data are represented as the mean ± SE. Bars with different letters (a, b, c, d, and e) denote means significantly different from each other ($P < 0.05$) as determined by Tukey's HSD.

11.4% on d 2 to 20.0% on d 8, $P < 0.05$). There were no significant differences between the two groups after 8–10 d of tannin consumption (Figure 3, d 8 PGG-naïve *vs*. d 2 PGG-habituated, $P > 0.05$).

Apparent Dry Matter and Nitrogen Digestibility. During the first 3 d on the 3% PGG diet, there was no significant change in either apparent DM or N digestibility (Figure 4, compare between groups on d 1a–3a, $P > 0.05$). However, as the rats became habituated to the 3% PGG diet, both apparent DM and N digestibility significantly decreased, with N digestibility affected more strongly. In the PGG-habituated group, there was a 7% decrease in the apparent DM digestibility from d 1a–3a to 8–10 ($P < 0.001$). For the PGG-naïve group, the decrease in apparent DM digestibility was 5% from d 1a–3a to 8–10 ($P < 0.05$). The PGG-habituated group had a 25% decrease in the apparent N digestibility from d 1a–3a to 8–10 ($P < 0.001$), while the PGG-naïve group had a 21% decrease in the apparent N digestibility from d 1–3 to 8–10 ($P < 0.05$).

On the basis of fecal proline and ^{14}C excretion, we calculated PRP and PGG fecal excretion. By our calculations, fecal PRP and PGG excretion accounts for a maximum of 24% of the reduction in apparent DM digestibility, and fecal PRP excretion accounts for a maximum of 38% of the reduction in apparent N digestibility that we measured in PGG-habituated rats (Table 3).

DISCUSSION

The ability of tannins to bind proteins has led to the widespread notion that dietary tannins reduce protein digestibility (Feeney, 1968; Robbins et al., 1987; Horigome et al., 1988; Hanley et al., 1992; Ortiz et al., 1994; Jansman et al., 1995). However we found that when rats consume PGG, a hydrolyzable tannin, for a few days, there is no significant effect on apparent dry matter and nitrogen digestibility. Only after consuming PGG for 8 d or more was there a significant decline in apparent dry matter and nitrogen digestibility (7 and 25%, respectively). We speculate that production of salivary PRPs, which in the rat are induced during the first 8–10 d of ingesting dietary tannin (Mehansho et al., 1983), could be responsible for observed reductions in digestibility seen during consumption of tannin-containing diets in some animals. Our idea contradicts the widely held hypothesis that PRP production protects animals from digestibility reduction (Mole et al., 1990; Robbins et al., 1991; McArthur et al., 1995).

We are not the first to report that the reduction in nitrogen digestibility observed when animals consume tannin is due to endogenous nitrogen loss and not due to direct decreases in the digestibility of dietary nitrogen (Glick and Joslyn, 1969; Mitjavila et al., 1977; Jansman et al., 1995). However, our study is the first to show a reduction in apparent nitrogen digestibility within the same animals across three different dietary situations: tannin-free diets, 3% PGG diet fed to

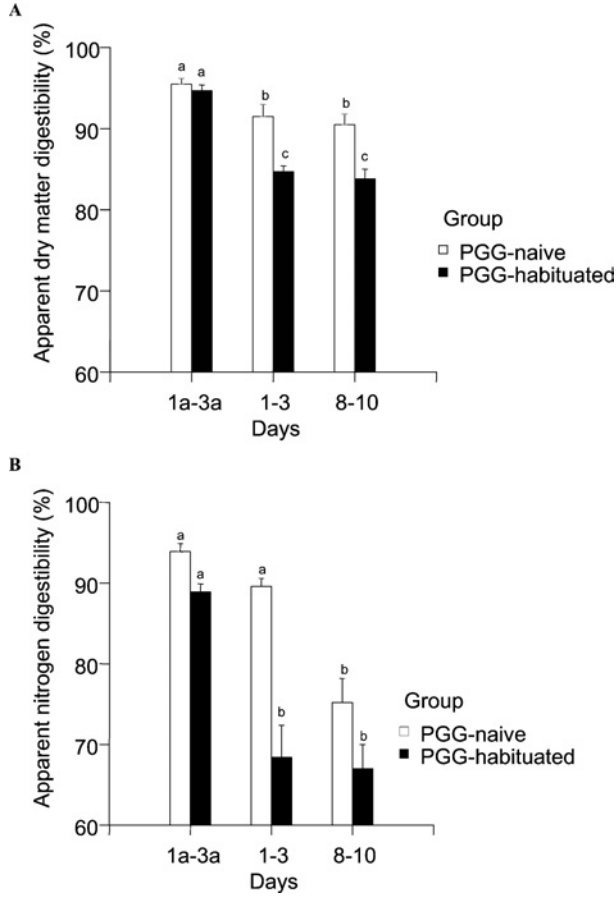

FIG. 4. PGG habituation *vs*. apparent diet digestibility. (A) Shows the 3-d average of apparent dry matter digestibility. (B) Shows the 3-d average of apparent nitrogen digestibility. The PGG-habituated group was placed on the 3% PGG diet starting d 1a, and had been eating the 3% PGG diet for 9 d by d 1. The PGG-naïve group was on the basal diet on d 1a–3a and was placed on the 3% PGG diet on d 1. Data are represented as the mean ± SE. Bars with different letters (a, b, c, d, and e) denote means significantly different from each other ($P < 0.05$) as determined by Tukey's HSD.

animals not producing PRP, and 3% PGG diet fed to animals that were producing PRP. We found that there was a significant reduction in apparent nitrogen and dry matter digestibility only when the rats were fed the 3% PGG diet for a long enough period to induce PRP secretion. PRP secretion did not mitigate the presumed digestibility reducing effects of PGG, because PGG itself had no effect

TABLE 3. THE PERCENT OF THE REDUCTION IN APPARENT DIET DIGESTIBILITY EXPLAINED BY CALCULATED FECAL EXCRETION OF PGG AND PRP

Group	Apparent digestibility (mean ± SD)	
	Nitrogen[a]	Dry matter[b]
PGG-naïve ($n = 5$)	21.6 ± 3.6	12.0 ± 3.1
PGG-habituated ($n = 5$)	38.2 ± 3.5	23.7 ± 5.7

[a] Based on fecal PRP excretion.
[b] Based on fecal PRP and PGG excretion.

on apparent digestibility. Instead PRP secretion may have increased the fecal loss of endogenous materials, thus reducing apparent digestibility, especially for nitrogen.

We calculated that fecal PRP excretion accounts for up to a maximum of 38% of the decrease in apparent nitrogen digestibility while fecal PRP and PGG excretion account for a maximum of only 24% of the decrease in apparent dry matter digestibility seen in PGG-habituated animals. Gastrointestinal mucous may comprise at least part of the remaining nitrogen and dry matter losses in the PGG-habituated animals, as suggested by the elevated fecal glucosamine and sialic acid noted in rats on a 1% tannic acid diet (Mitjavila et al., 1977). An increase in mucous production and secretion can occur after the integrity of the digestive epithelium is impaired (Deplancke and Gaskins, 2001). The mucous may protect the gastrointestinal tract from oxidative damage incurred due to the pro-oxidative activities of some polyphenolics (Mitjavila et al., 1977; Ortiz et al., 1994; Long et al., 2000). The PAR-2 pathway controls both mucous production in gastrointestinal cells and secretion by the salivary glands (Kawabata et al., 2001), leading us to speculate that dietary tannin may use a single pathway to induce both PRP and mucous secretion.

We believe that PRPs do play an important role in protecting animals from the effects of hydrolyzable tannins that are not related to digestibility. This hypothesis is supported by the increase in food consumption and weight gain observed after habituation to dietary tannin compared to the low intakes and weight losses recorded for tannin-naïve animals when first introduced to dietary tannin. Low intakes and consequent weight loss may be due to the astringent taste of PGG-containing diets, which may be alleviated by complexation of the polyphenols to PRPs (McArthur et al., 1995). By modifying astringent tastes, salivary PRPs may provide an important mechanism for animals to ingest sufficient amounts of otherwise unpalatable tannin-rich food and, thus, utilize these foods as part of their diets.

PRPs may also minimize toxicity of PGG by minimizing absorption of the polyphenolic or its hydrolysis products. Our data did not indicate whether the

unexcreted PGG was absorbed or was hydrolyzed in the GI tract. Hydrolysis would release glucose that could be metabolized by either the rat or its gut microflora to produce energy, but absorption of either gallic acid or intact PGG would impose the metabolic costs of detoxification and excretion (Booth et al., 1959; Zong et al., 1999; Niho et al., 2001). That metabolic cost could be minimized by excretion of intact PRP–PGG complexes in the feces. Although only a maximum of 20% of the ingested PGG was excreted in the feces of PGG-habituated animals, this is a three- to fourfold increase in excretion over the PGG-naïve animals, and could reflect significant reduction in metabolic costs.

In laboratory rats, induction of PRPs conferred the benefit of a three- to fourfold increase in the amount of PGG excreted in the feces but it came with the cost of increasing the loss of endogenous mass and nitrogen measured as a decline in apparent dry matter digestibility by up to 7% and apparent nitrogen digestibility by up to 25%. Unbound hydrolyzable tannins could cause tissue damage within the intestinal tract (Mitjavila et al., 1977; Ortiz et al., 1994), target site toxicity post absorption (Murdiati et al., 1991; Niho et al., 2001), and create a metabolic cost for the detoxification and excretion of absorbed tannin components (Lindroth and Batzli, 1983; Thomas et al., 1988; Iason and Murray, 1996). In contrast, a reduction in apparent nitrogen and dry matter digestibility may only be detrimental in situations where either nitrogen or overall energy is limiting. By increasing endogenous losses of nitrogen through the secretion of PRPs, animals on a nitrogen-limited diet may alter their overall nitrogen balance, reallocating nitrogen from growth and reproduction to PRP production (Meyer and Richardson, 1993). Wild animals could counteract the loss of nitrogen by eating more, which may come with the cost of an increased risk of predation. The rats in this study were fed a diet containing 20% protein. The minimum protein requirement for laboratory rats for growth is 15% (NRC, 1995) and, therefore, nitrogen was not limiting. Thus, the cost of an increase in endogenous nitrogen loss would have been easily paid by the rats, well worth the increased protection the PRP may have provided from potential tissue damage and target site toxicity post absorption.

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SEASONAL VARIATION IN THE CONTENT OF HYDROLYZABLE TANNINS, FLAVONOID GLYCOSIDES, AND PROANTHOCYANIDINS IN OAK LEAVES

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Abstract—Oaks have been one of the classic model systems in elucidating the role of polyphenols in plant–herbivore interactions. This study provides a comprehensive description of seasonal variation in the phenolic content of the English oak (*Quercus robur*). Seven different trees were followed over the full course of the growing season, and their foliage repeatedly sampled for gallic acid, 9 individual hydrolyzable tannins, and 14 flavonoid glycosides, as well as for total phenolics, total proanthocyanidins, carbon, and nitrogen. A rare dimeric ellagitannin, cocciferin D_2, was detected for the first time in leaves of *Q. robur*, and relationships between the chemical structures of individual tannins were used to propose a biosynthetic pathway for its formation. Overall, hydrolyzable tannins were the dominant phenolic group in leaves of all ages. Nevertheless, young oak leaves were much richer in hydrolyzable tannins and flavonoid glycosides than old leaves, whereas the opposite pattern was observed for proanthocyanidins. However, when quantified as individual compounds, hydrolyzable tannins and flavonoid glycosides showed highly variable seasonal patterns. This large variation in temporal trends among compounds, and a generally weak correlation between the concentration of any individual compound and the total

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concentration of phenolics, as quantified by the Folin–Ciocalteau method, leads us to caution against the uncritical use of summary quantifications of composite phenolic fractions in ecological studies.

Key Words—*Quercus robur*, hydrolyzable tannins, ellagitannins, flavonoid glycosides, proanthocyanidins, HPLC, quantification of phenolics, biosynthetic pathways, compound-specific patterns.

INTRODUCTION

Oaks in the genus *Quercus* have long been a popular target for ecologists studying the chemical interplay between plants and herbivorous insects (e.g., Feeny, 1970; Faeth, 1986; Rossiter et al., 1988; Tikkanen and Julkunen-Tiitto, 2003). One of the earliest and still most influential ecological explorations of oak leaf chemistry was conducted by Feeny (1970). In his seminal paper, he related the phenology of an oak-feeding moth (*Operopthera brumata*) to seasonal variation in the chemical contents of oak leaves. By quantifying relatively crude fractions of phenolic compounds, he inferred that the nutritional value of oak leaves declines throughout the summer, and that this may be the ultimate factor causing spring-feeding in *O. brumata*.

Phenolics may well play a central part in the oak's defense against its herbivores (e.g., Schultz and Baldwin, 1982; Rossiter et al., 1988). However, past phytochemical and experimental studies leave no doubt that individual phenolic compounds vary substantially with respect to biological activity (Zucker, 1983; Ozawa et al., 1987; Clausen et al., 1990; Ayres et al., 1997; Feldman et al., 1999; Kilkowski and Gross, 1999; Kraus et al., 2003) and that even chemically closely related compounds may encounter a different fate in the digestive tract of an insect (e.g., Salminen and Lempa, 2002). Hence, to understand the interplay between the oak and its herbivores, it seems preferable to measure the concentrations of individual compounds. The quantification of composite phenolic fractions will only make sense if (a) concentrations of individual phenolics vary in concert and (b) the quantified compounds have matching biological activities—presumably a rare combination in real organisms.

So far, a relatively large number of compound-specific studies have investigated the hydrolyzable tannin composition of oak wood (e.g., Masson et al., 1994; Viriot et al., 1994; Conde et al., 1998; Mosedale et al., 1998; Fernández de Simón et al., 1999; Mämmelä et al., 2000; Cadahía et al., 2001). This interest has primarily been spurred by the needs of the wine industry, as most wine barrels are made of English oak (*Quercus robur*) or sessile oak (*Q. petraea*). Of the named hydrolyzable tannins, the heartwood of *Q. robur* has been found to contain castalagin, vescalagin, grandinin, and roburins A–E (Hervé du Penhoat et al., 1991a,b; Masson et al., 1994; Vivas et al., 1995).

Substantially less attention has been aimed at the phenolic composition of oak leaves. A handful of earlier studies have shown the foliage of *Q. robur* to contain pedunculagin, castalagin, vescalagin, casuarictin, and flavonol glycosides (Scalbert and Haslam, 1987; Scalbert et al., 1988; Grundhöfer et al., 2001). However, this information has only rarely been utilized in ecological studies that try to relate the polyphenolic composition of oak leaves to the performance of folivorous insects. Most often, only total phenolics or the total concentrations of summary tannin groups have been quantified (e.g., Feeny, 1970; Faeth, 1986; Rossiter et al., 1988; Lill and Marquis, 2001; Abrahamson et al., 2003; Forkner et al., 2004; but see Tikkanen and Julkunen-Tiitto, 2003), and how well such composite measures will reflect variation at the level of individual compounds has remained an open question.

In this paper, we provide the first comprehensive description of seasonal variation in the phenolic contents of oak leaves as quantified at the level of both individual compounds and two summary groups—total phenolics and total proanthocyanidins. For the first time, we also report the presence of a rare dimeric ellagitannin, cocciferin D_2, in the foliage of *Q. robur* and propose a biosynthetic pathway for its formation. On the basis of seasonal patterns observed in individual compounds, we ask: (1) To what extent can seasonal variation in the concentrations of individual compounds be described by a single summary measure such as the total concentration of phenolic compounds? (2) How well is variation among compounds explained by biochemical connections—will the knowledge of central metabolic pathways help us understand temporal changes in concentrations of individual compounds? (3) What does variation in phenolic content and variation in the total content of carbon and nitrogen reveal about changes in the nutritional value of oak leaves over the season?

METHODS AND MATERIALS

Study Object. The deciduous English oak is native to Europe and Western Asia (Jalas and Suominen, 1976). In Finland, its natural distribution is limited to the southernmost part of the country, where new oak leaves are produced in late May and early June (Hoffman and Lyr, 1973; Niemelä and Haukioja, 1982). Although the period of active leaf production is relatively short and well-synchronized in oak as compared to other tree species (Niemelä and Haukioja, 1982), its onset may vary by several weeks between both years and trees (cf. Crawley and Akhteruzzaman, 1988). Within trees, leaf production is apparently more synchronized on short shoots than on actively growing long shoots (Erkki Haukioja, personal communication, 2003). As a result, leaves on short shoots will be of similar age throughout the summer; only in some oak individuals is there a second growth period in August, resulting in so-called "lammas shoots" with new leaves. All leaves typically senesce in October, but variation among trees and

years is again substantial. While most leaves are shed in the autumn, some trees retain a high proportion of dead leaves until the following spring.

Study Site. In the spring of 2001, seven oaks were selected within a small (about 0.1 ha) stand of 21 oaks growing at the Haapastensyrjä Tree Breeding Center (60° 36′ N, 24° 26′ E), run by the Finnish Forest Research Institute. These oaks had been planted in 1978 and were now approximately 7–8 m in height. Each tree was originally created by grafting a small oak twig to a sapling reared from a randomly collected acorn. Any twigs produced by the original sapling were later pruned, and hence the current canopy of the oak consists of the grafted genotype only. The grafted twigs had been collected in oak stands across the full Finnish range of the English oak.

Leaf Sampling. Leaf samples were collected from each of the seven oaks on 11 dates throughout the summer of 2001. The sampling dates ranged from May 29, which was the 1st d when each tree had leaves larger than 1 cm, to September 26, when the leaves were already senescing. Individual sampling dates were May 29, June 7, June 15, June 24, July 7, July 16, July 27, August 16, August 31, September 13, and 26. All samples were collected between 8 and 12 A.M. A similar number of leaves (7–30, depending on the date and leaf size) were randomly collected from the lower branches of each tree. To reduce variation among leaves within a sample, we specifically avoided the hard and waxy "sun leaves" of the outer canopy (cf. Feeny, 1970) as well as leaves on actively growing long shoots. Leaves were sealed in polyethylene bags in the field and placed into a cooler with ice. Upon return to the laboratory, they were air-dried for 3 d at room temperature in a ventilated fume cupboard, and samples were weighed and subsequently stored at –18°C until ground into a fine powder before extraction. Although air-drying may not be the optimal drying method, it is known to alter the levels of hydrolyzable tannins and flavonoid glycosides only minimally in birch leaves (Salminen, 2003, unpublished data) or oak leaves (Salminen, unpublished pilot study) as compared to levels observed in freeze-dried samples. For each sample, the average biomass per leaf was calculated by dividing the total weight of the sample by the number of leaves it contained.

Extraction. Dried and ground oak leaves (200 mg per sample) were extracted four times (4 × 1 hr) with 70% aqueous acetone (4 × 8 ml) on a planary shaker. The freeze-dried aqueous phase of the extract was dissolved in water (3 × 2 ml); the supernatant of the centrifuged (10 min at 2000 *g*) sample was filtered through a 0.45-μm PTFE filter and kept frozen at –20°C until analyzed with HPLC-DAD or HPLC–ESI-MS.

Analysis of Phenolics with HPLC-DAD and HPLC–ESI-MS. HPLC-DAD analysis of oak leaf extracts was performed at 280 and 349 nm with Merck-Hitachi's LaChrom HPLC system (Merck-Hitachi, Tokyo, Japan). Column and chromatographic conditions were as described earlier (Salminen et al., 1999),

except that 0.1 M H_3PO_4 was replaced with 0.05 M H_3PO_4. Phenolic compounds were quantified using pedunculagin, 1-*O*-galloylglucose, gallic acid, quercetin, and kaempferol as external standards.

HPLC–ESI-MS analysis was performed using a Perkin-Elmer Sciex API 365 triple quadrupole mass spectrometer (Sciex, Toronto, Canada) equipped with an ion-spray interface. The HPLC system consisted of two Perkin-Elmer Series 200 micro pumps (Perkin-Elmer, Norwalk, CT, USA) connected to a Series 200 autosampler. The column used and chromatographic and ESI-MS conditions were as described previously (Salminen et al., 1999).

Isolation and Identification of Cocciferin D_2. Cocciferin D_2 (120 mg) was isolated from oak leaves with a combination of Sephadex LH-20 (40 × 2.5 cm i.d.) and Merck LiChroprep RP-18 (44 × 3.7 cm i.d., 40–63 μm) columns following the methods outlined by Salminen et al. (1999, 2001). Part of the pure isolate was partially hydrolyzed in mild conditions (40°C water, 1 hr) and the reaction products analyzed by HPLC-DAD and HPLC–ESI-MS. The NMR spectra of Cocciferin D_2 were acquired using a JEOL JNM-A-500 spectrometer operating at 500.16 MHz for ^{1}H and 125.78 MHz for ^{13}C. Spectra were recorded at 25°C using acetone-d_6 as a solvent. In addition to standard proton and carbon spectra, DEPT, DQF-COSY, HMQC, and HMBC spectra were measured.

Analyses of Total Phenolics, Proanthocyanidins, Carbon, and Nitrogen. The total phenolic content of the extracts was determined by a modification of the Folin–Ciocalteau method (Nurmi et al., 1996), using a Perkin-Elmer Lambda 12 UV–VIS spectrometer (Norwalk, CT, USA). Three replicates of each sample were analyzed and their average was used as the final reading. A standard curve was prepared on the basis of known concentrations of gallic acid. The total content of proanthocyanidins was measured with the butanol-HCl assay as in Ossipova et al. (2001). Again, measurements were based on the average reading of three replicate samples, and a standard curve prepared on the basis of known concentrations of purified birch leaf proanthocyanidins. The total concentration of carbon and nitrogen in the leaves was performed with a Perkin-Elmer Series II CHNS/O Analyzer 2400 (Norwalk, CT, USA). A subset of six samples was included in this analysis, corresponding to sampling dates May 29, June 7, June 15, July 7, August 16, and September 26.

Data Analysis. Seasonal changes in leaf chemistry were described by visual plots of compound-specific patterns. To further evaluate the extent to which the concentrations of different compounds vary in unison among sampling dates, we calculated simple Spearman rank correlation coefficients (r_S). All trees were first ranked within compounds and sampling dates, from the tree richest in this particular compound to the tree with the lowest concentration. Then, two types of comparisons were made on the basis of samples taken on the date with the highest phenolic readings (May 29). First, to illustrate the concordance between

the concentration of an individual compound and the pooled concentration of larger phenolic groups, correlation coefficients were calculated for the rank of the compound in question and the rank of the pooled concentrations of all individual compounds as quantified by HPLC. Second, to depict the consistency between the concentration of an individual compound and the total concentration of phenolics as quantified by a summary method, correlation coefficients were calculated for a tree's rank for the compound in question and its rank for total phenolics as quantified by the Folin–Ciocalteau method. From an applied perspective, what we ask is specifically: "if we know the precise concentrations of individual compounds, will patterns at the level of total phenolics summarize patterns at the level of individual compounds?"

RESULTS

Characterization of Phenolic Compounds. Twenty-four phenolic compounds were detected in the HPLC-DAD analyses. From their UV spectra, two were preliminarily identified as gallic acid or its derivatives (compounds **1**–**2**; Salminen et al., 1999), and eight as ellagitannins (**3**–**10**; Salminen et al., 1999). The rest of the compounds were classified on the basis of both UV and mass spectral characteristics as quercetin (**11**–**19**; Ossipov et al., 1995, 1996) or kaempferol glycosides (**20**–**24**; Ossipov et al., 1995, 1996).

Since tannins are generally supposed to play a more important role in plant–herbivore interactions than flavonoid glycosides, we focused more on the identification of compounds **1**–**10** than on **11**–**24**. On the basis of their retention times (R_ts; compared to those given in the literature) and molecular masses recorded from a negative ion HPLC–ESI-MS run, the structures of **1**–**5** were identified as gallic acid (**1**, 6.7 min, 170 g/mol), 1-*O*-galloylglucose (**2**, 6.4 min, 332 g/mol), tellimagrandin II (**3**, 19.2 min, 938 g/mol), casuarictin (**4**, 18.0 min, 936 g/mol), and pedunculagin (**5**, 9.4 and 11.7 min, anomeric mixture, 784 g/mol; Salminen et al., 2001). Ellagitannin **6** (14.3 min, 936 g/mol) could be either stachyurin or casuarinin; only one of these isomers was detected in the *Q. robur* extract, thus making it impossible to utilize the R_t data of Okuda et al. (1982) for differentiating between the two. In contrast, the other two isomeric ellagitannins, i.e., **7** (8.4 min, 934 g/mol) and **8** (10.2 min, 934 g/mol), were clearly detected and identified as vescalagin and castalagin, respectively (Fernández de Simón et al., 1999; Zhentian et al., 1999). At this point, the structures of two ellagitannins, i.e., **9** (7.6 min, 1102 g/mol) and **10** (15.2 min, 1868 g/mol), remained unresolved.

The mild hydrolysis of **10** yielded **5** and an unidentified ellagitannin with a molecular mass of 1084 g/mol; thus, **10** was shown to be a dimeric ellagitannin

with **5** as one of the monomeric constituents. The whole chemical structure of **10** was unravelled by a combination of NMR experiments. All proton and carbon chemical shifts, as well as the proton–proton coupling constants, matched perfectly with those reported for cocciferin D_2, an ellagitannin recently found in the leaves of *Q. coccifera* and *Q. suber* (Ito et al., 2002). Therefore, **10** was identified as cocciferin D_2, now detected for the first time in leaves of *Q. robur*. This dimeric ellagitannin consists of two monomeric units, i.e., **5** and castavaloninic acid. The latter of these compounds has a molecular mass of 1102 g/mol, corresponding to that of monomeric ellagitannin **9**. Interestingly, according to Yoshida et al. (1992), the valoneoyl group of castavaloninic acid may undergo lactonization (loss of water) upon hydrolysis resulting in a depsidone molecule having a molecular mass of 1084 g/mol (see also hydrolysis of **10** above). This is exactly the same as that of a significant fragment of **9** produced in negative ion HPLC–ESI-MS, which is known to fragment hydrolyzable tannins in a manner similar to chemical hydrolysis (cf. Salminen et al., 1999, 2001; Salminen, 2002). For these reasons, **9** was identified as castavaloninic acid.

A Biosynthetic Pathway for the Hydrolyzable Tannins of Oak Leaves. By examining the relationships among the chemical structures of individual tannins identified in this study, we propose a biosynthetic pathway for their formation. It is well accepted (as reviewed by Gross, 1999) that the first compound in the general hydrolyzable tannin pathway, **2**, is formed from **1** and UDP-glucose, and that the galloylations then continue consecutively and position-specifically to finally yield 1,2,3,4,6-penta-*O*-galloylglucose. On the other hand, the formation of the first ellagitannin of the pathway, **3**, directly from pentagalloylglucose was proven only recently (Niemetz et al., 2001). The subsequent biosynthetic steps from **3** onwards have not been experimentally proven, but it is generally assumed that e.g., **4** is a product of further oxidative coupling of two spatially adjacent galloyl groups of **3** (see, e.g., Helm et al., 1999). Furthermore, it has been suggested that the C-glycosidic ellagitannins **7** and **8** are formed from **5**, and that this step contains at least stachyurin and casuarinin (**6**) as intermediates (see also Okuda et al., 1982; Hatano et al., 1986; Haslam, 1992; Vivas et al., 1995; Helm et al., 1999). We are not aware of any biosynthetic speculations about the origin of **9** and **10**, but on the basis of their structures it seems safe to assume that **9** is formed by galloylation at the hexahydroxydiphenoyl (HHDP) group of **8**, and that **10** is a product of dimerization of **5** and **9**. On the basis of these considerations, we arrive at the biosynthetic pathway depicted in Figure 1.

Seasonal Changes in Phenolic Contents. Throughout the summer, the phenolic contents of oak leaves were dominated by hydrolyzable tannins (Figure 2A and B). Although the pooled concentration of all hydrolyzable tannins declined by 54% between late May and September, their total concentration still overshadowed that of proanthocyanidins by a factor of 7.7 on September 26. The concentrations

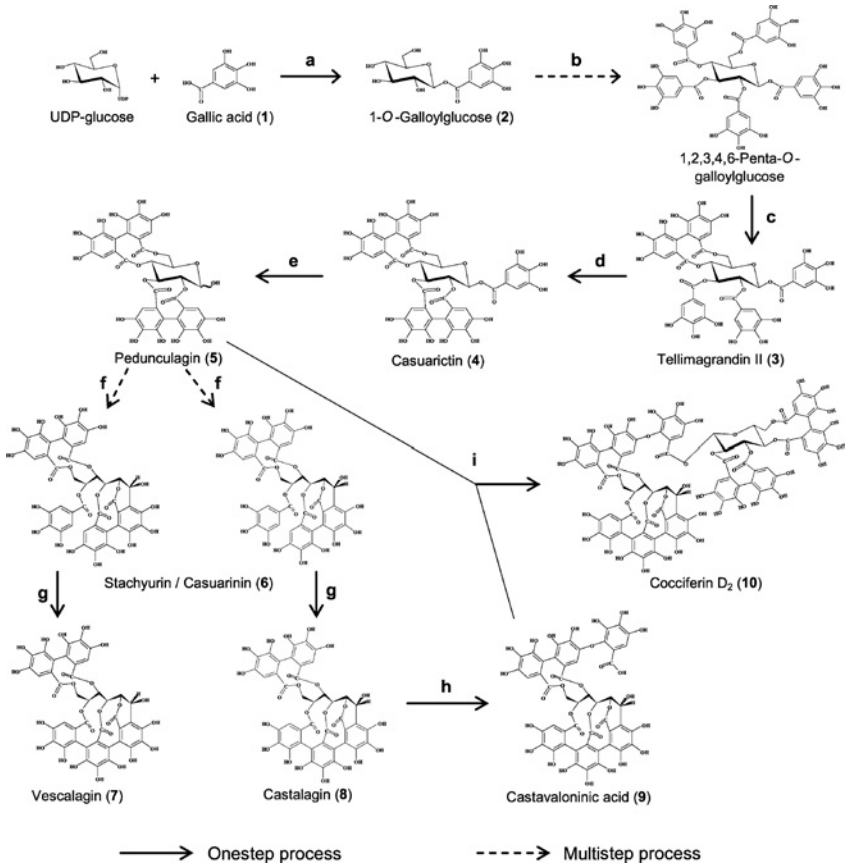

FIG. 1. The proposed biosynthetic pathway for the formation of ellagitannins in leaves of *Quercus robur* L. The biosynthetic steps involve (**a**) galloylation at C-1 of glucose; (**b**) four consecutive galloylation steps; (**c**) oxidative coupling between galloyl groups at C-4 and C-6; (**d**) oxidative coupling between galloyl groups at C-2 and C-3; (**e**) cleavage of galloyl group at C-1; (**f**) glucopyranose ring opening at C-1, followed by galloylation at C-5; (**g**) oxidative coupling between benzene rings at C-4 and C-5; (**h**) further galloylation thus forming a valoneoyl group at C-4/C-5; and (**i**) dimerization of pedunculagin and castavaloninic acid to form cocciferin D_2.

of proanthocyanidins showed an opposite seasonal pattern, steadily increasing over the summer from undetectable levels in the young leaves to an average of 10.8 mg/g in old leaves.

The most rapid changes in leaf chemistry occurred during the early part of the summer, as the oak leaves grew and matured (Figure 2). In the majority

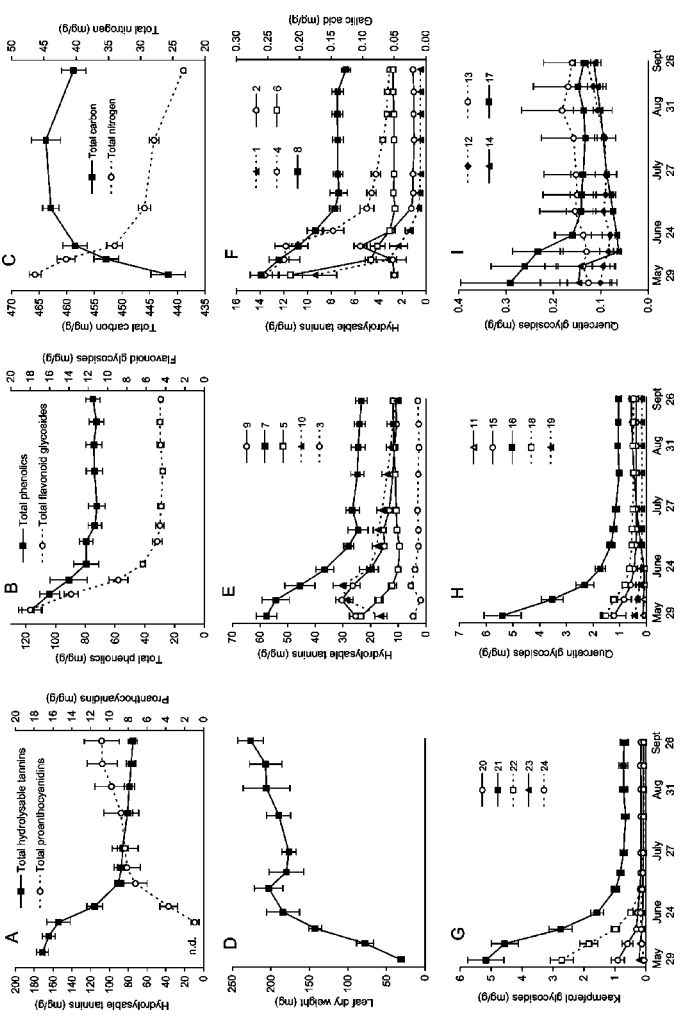

FIG. 2. Seasonal variation in leaf traits. Each data point represents the mean of seven trees, and vertical bars show standard errors. (A) Concentrations of total hydrolyzable tannins (sum of compounds **2**–**10**) and proanthocyanidins (quantified by the butanol-HCl assay). (B) Concentrations of total phenolics (quantified by the Folin–Ciocalteau assay) and flavonoid glycosides (sum of compounds **11**–**24**). (C) Total concentrations of carbon and nitrogen. (D) Biomass per dried oak leaf. (E and F) Concentrations of individual hydrolysable tannins and gallic acid. (G) Concentrations of individual kaempferol glycosides. (H and I) Concentrations of individual quercetin glycosides. Compounds numbered as in Figure 1.

of individual compounds, there was a distinct peak in concentration around the time of maximum leaf expansion (Figure 2), followed by an extended period of relatively stable values. After July 7, when the leaves had reached their final size, their phenolic composition remained more or less unchanged until leaf senescence (Figure 2). However, when inspected in more detail, different seasonal patterns emerge among individual compounds, with a few compounds peaking later in the season than the rest (notably **2**, **9**, and **10** among the tannins; Figure 2E–I).

Interestingly, the maximal concentrations of many phenolic compounds in late May and early June coincide with a minimum in the total amount of carbon, followed by a continuous buildup of carbon over the course of the summer (Figure 2C). For nitrogen, the pattern was exactly the opposite, with the level steadily decreasing over the summer (Figure 2C).

Consistency Among Individual Compounds and Larger Phenolic Groups. Variation at the level of total phenolic content did not capture variation at the level of individual compounds: a sample rich in total phenolics, or in the pooled concentration of all individual compounds, was not necessarily rich in any individual compound (Figure 3). Some of the correlations between the concentration of an individual compound and the total concentration of a larger phenolic group are trivial because we are comparing an element to a sum of which it is a part. In the absence of any correction for multiple tests, chance alone is also expected to render one in every 20 results "significant." Still, among correlations between individual compounds and total phenolic content, only one out of 48 r_S values was "significant," and most values were relatively low or even negative (Figure 3). At the level of the pooled concentrations of chemically similar compounds, only the concentration of hydrolyzable tannins was significantly correlated with the pooled concentration of all individual compounds (and perfectly so; $r_S = 1.0$). Finally, our two different measures of total phenolic contents—"total phenolics" as quantified by the Folin–Ciocalteau method and "pooled phenolics" as calculated by pooling the HPLC readings of individual compounds—were not significantly correlated with each other ($r_S = 0.71$, $N = 7$, $P = 0.07$). Hence, we conclude that the rough quantification of a composite phenolic fraction tells us virtually nothing about how different trees rank compared to each other in terms of individual compounds.

DISCUSSION

Oaks have formed one of the classic model systems in elucidating the role of polyphenols in plant–herbivore interactions. This study provides, to our knowledge, the most elaborate description of temporal variation in the phenolic contents of oak leaves conducted so far. As such, it adds substantial detail to the already classical image of oak leaf chemistry drawn by Feeny in 1970. However, several

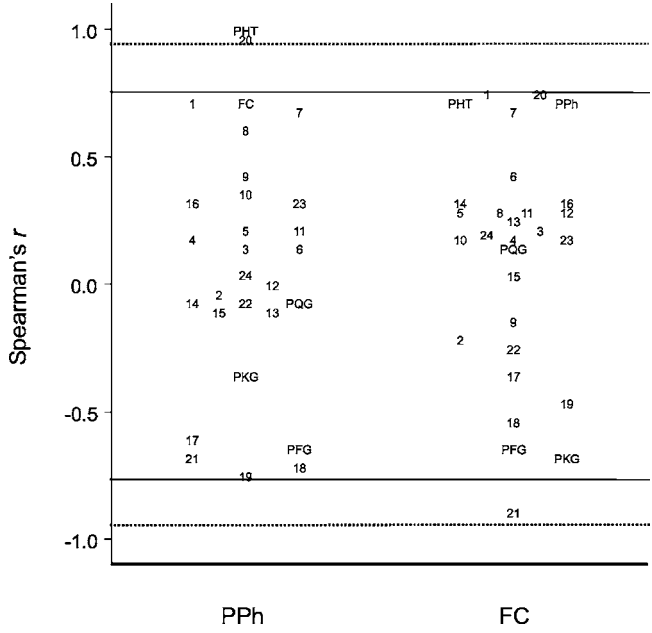

FIG. 3. Spearman rank correlations (r_S) among different phenolic fractions in samples from May 29. PPh (i.e., Pooled Phenolics) shows the consistency in rank (r_S) between the concentration of individual compounds and the pooled concentration of all individually quantified phenolic compounds; FC (i.e., Folin–Ciocalteau) shows r_S values for the compound in question compared to Folin–Ciocalteau readings of total phenolics. For each data point, the number shows the identity of the compound (numbers as in Figures 1 and 2). Data points labelled with letters refer to pooled concentrations of different phenolic subgroups; PFG = Pooled Flavonoid Glycosides (**11**–**24**), PHT = Pooled Hydrolyzable Tannins (**2**–**10**), PQG = Pooled Quercetin Glycosides (**11**–**19**), and PKG = Pooled Kaempferol Glycosides (**20**–**24**). For all values of r_S, $N = 7$ trees. The horizontal bars at the top and bottom of each panel shows the critical r_S for $P < 0.05$ before (solid line) and after (stippled line) Bonferroni correction for 29 individual tests.

discrepancies with Feeny show the need for some substantial specifications to current descriptions of oak leaf chemistry.

In his seminal account of seasonal variation in oak leaf quality, Feeny (1970, p. 574) reported a general increase in the tannin contents of oak leaves over the course of the summer. On the basis of two-dimensional paper chromatography, he attributed this pattern to a progressing dominance of proanthocyanidins over hydrolyzable tannins, the levels of which appeared to remain approximately constant over time. Our results strongly contrast with those findings. First, we

found hydrolyzable tannins, not proanthocyanidins, to be the dominant group of phenolic compounds over the full course of the growing season. The peak levels of hydrolyzable tannins were strikingly high—while heartwood of *Q. robur* can contain up to 10% of ellagitannins by weight (Scalbert et al., 1988), young oak foliage contained levels as high as ca 18% (see Figure 2A). Second, the concentration of hydrolyzable tannins did not remain stable over time, but dropped to less than half between late May and September. Early-summer maxima in the levels of hydrolyzable tannins have been reported from *Q. robur* (e.g., Tikkanen and Julkunen-Tiitto, 2003), from other oak species (e.g., Faeth, 1986; Rossiter et al., 1988; Maufette and Oechel, 1989), and from other trees (e.g., Riipi et al., 2002). In all these cases, the early peak in hydrolyzable tannins is associated with a late-summer peak in condensed tannins, suggesting a common pattern across species and upsetting Feeny's (1970) initial notion. Yet, there is one important difference between our material and Feeny's: where Feeny focused on the upper sun leaves of the canopy, we collected our leaf samples on the lower branches of the trees. As light levels have been found to affect foliar phenolics (e.g., Dudt and Shure, 1994), part of the observed difference may be due to differences in leaf exposure. This calls for further analyses of seasonal changes in different parts of the canopy.

Feeny (1970) focused on seasonal patterns at the level of summary phenolic groups. When we examined patterns at the level of individual compounds, temporal changes at the level of pooled phenolic contents were found to mask variation in the concentration of individual hydrolyzable tannins and flavonoid glycosides over time (Figures 2 and 3). The ultimate, evolutionary reasons for the observed variation in compound-specific patterns are largely unknown, as consensus has yet to be reached even regarding the exact biological roles of hydrolyzable tannins and flavonoid glycosides (cf. Appel, 1993; Close and McArthur, 2002). Proximate reasons are better understood, as seasonal changes in the concentration of individual hydrolyzable tannins can sometimes be mapped onto proposed or established biogenetic pathways (cf. Hatano et al., 1986; Salminen et al., 2001). In *Q. robur*, differences in the way individual compounds changed over time appeared closely connected to their biosynthetic relationships. First, compound **2** was the only galloylglucose present in oak leaves in levels detectable with HPLC-DAD. This was surprising, since the biosynthetic pathway of hydrolyzable tannins also contains di-, tri-, tetra-, and pentagalloylglucoses before the first ellagitannin, i.e., **3** (Figure 1; cf. Gross, 1999; Niemetz et al., 2001; Salminen et al., 2001). Evidently the synthesis of ellagitannins in oak is effective enough not to let these galloylglucose intermediates accumulate in detectable amounts. Consistent with this view of active ellagitannin synthesis, the concentration of **2** (i.e., the compound from which a galloyl group is utilized in the formation of other galloylglucoses and ellagitannins therefrom) decreased dramatically after June 15 (see Figure 2F).

Second, temporal variation in the concentrations of compounds **7**, **8**, **9**, and **10** appears intimately interrelated (Figure 2). Although all the other ellagitannins showed almost linear reductions in their concentrations already from May 29 onwards, ellagitannin **9**, i.e., the monomeric building block of **10**, showed an increase until June 7, and its biosynthetic successor **10** until June 15. Simultaneously, the concentration of **8**, the building block of **9**, showed a more steep decrease than that of **7**. This was presumably because **8** was utilized further in the biosynthesis of oak leaf ellagitannins (for formation of **9** and **10**) unlike its isomer **7** (see Figure 1). For the same reason, the concentration of **7** was approximately four times as high as that of **8** throughout the season (e.g., May 29; 57.6 mg/g vs. 14.0 mg/g). However, by summing the concentration of **8** with that of its biosynthetic successors **9** and **10**, almost equal values were obtained as with **7** only (e.g., May 29; 55.8 mg/g vs. 57.6 mg/g, respectively). This implies that oak leaves invest a closely similar amount of resources in the production of **7** and **8**, although this cannot be seen in their foliar levels as such. Importantly, the potential for such "invisible investment" in a given compound suggests that firm conclusions on the specific pattern of resource allocation in oak trees cannot be reached until compounds are quantified individually and biosynthetic pathways unravelled.

Considering the proposed biogenetic pathway for *Q. robur* leaves (Figure 1), it is intriguing to note that it does not contain grandinin or roburins A–E—compounds that occur as biosynthetic successors of **7** and **8** in the heartwood of this species (Hervé du Penhoat et al., 1991a,b; Masson et al., 1994; Vivas et al., 1995). At the same time, ellagitannins **9** and **10** have not been encountered in the heartwood, but have now been found for the first time in leaves of *Q. robur*. Therefore, it is evident that enzymatic systems controlling the directions of the ellagitannin pathways onwards from e.g., **7** and **8** differ even between plant parts, not just among plant species as stated by Hatano et al. (1992). Interestingly, Scalbert et al. (1988) noted that the proportions of **7** and **8** vary between samples from different parts of *Q. robur*, the former being predominant in the leaves and the latter in the wood. However, they were unable to identify one major peak from the HPLC chromatogram of the leaf extract, thus possibly neglecting the presence of the dimeric ellagitannin **10**. If the unidentified peak was indeed due to **10**, that might explain the lower level of **8** in the foliage. Nevertheless, the observed differences in ellagitannin synthesis pathways caution against uncritical generalizations among different plant tissues when interpreting, e.g., patterns of seasonal change in phenolic composition.

Variation in oak leaf chemistry will affect a broad range of oak-associated taxa, including herbivorous mammals and arthropods, pathogenic and endosymbiotic fungi, and other microorganisms. Among Lepidoptera alone, more than 200 species feed on oak leaves—a figure higher than for any other European tree species (Feeny, 1970). The majority of these species attack young oak leaves in the spring (Feeny, 1970; Niemelä and Haukioja, 1982), and the ratio between oak

specialists and generalists similarly peaks early in the season (Niemelä, 1983). Feeny (1970) attributed this pattern to a clearcut decline in the nutritional quality of oak leaves over the course of the summer, largely because of an accumulation of phenolics in general and proanthocyanidins in particular, is presumed to precipitate proteins in the digestive system of the larva (Feeny, 1970; Niemelä, 1983; cf. Herms and Mattson, 1992). This view is partly upset by the current results, as the total concentrations of phenolics clearly decreased over time, and the concentration of hydrolyzable tannins was found to dominate over proanthocyanidins. Hence, if there is a general decline in oak leaf quality over time, it can hardly be linked to crude changes in phenolic contents—if it were, we would instead expect an increase (cf. Figure 2A and B). Given different seasonal patterns both among proanthocyanidins and hydrolyzable tannins as groups, and among individual compounds within these groups, the total effect of phenolics on seasonal variation in oak leaf quality will also depend on the relative biological activities of each individual compound.

Nevertheless, under no circumstances will herbivore performance be determined by phenolic contents alone—as emphasized by Haukioja (2003), the impacts of phenolics should be interpreted against the background of seasonal changes in crucial nutrients. Our elemental analysis showed that the nitrogen concentration of leaves declined by more than 50% between late May and September, suggesting a rapid decline in the availability of proteins and free amino acids. At the same time, the carbon contents of the leaves increased, indicating a buildup of lignin causing increased toughness of the leaves. Changes in nitrogen content and leaf toughness were also observed by Feeny (1970) and may interact with other attributes of the leaves such as water content (Mattson and Scriber, 1987; Haukioja et al., 2002; Henriksson et al., 2003) in determining their nutritional quality. In the end, a wealth of physical and biochemical factors may influence the quality of growing leaf tissue from an herbivore's perspective (Kause et al., 1999), and much work remains to be done before the chemical contents of an oak leaf can be linked to its perceived nutritional value.

From a methodological perspective, the observed idiosyncrasies among individual compounds cast some doubt on the common use of so-called "total methods"—e.g., Folin–Ciocalteau for total phenolics, the sodium nitrite method for total ellagitannins (Wilson and Hagerman, 1990), and the rhodanine method for total gallotannins (Inoue and Hagerman, 1988). Clearly, when concentrations of individual compounds vary more or less independently of each other, any method quantifying their pooled concentration will be a poor descriptor of patterns at the level of single compounds. This fact was graphically demonstrated by a general lack of correlation between the contents of individual compounds and total phenolics as quantified from the same samples (Figure 3).

Furthermore, "total methods" do not provide an unbiased measure even of the sum of individual compounds, as shown by a discrepancy between the

pooled amount of individual hydrolyzable tannins, flavonoid glycosides, and condensed tannins on the one hand, and "total phenolics" as quantified by the Folin–Ciocalteau method on the other (Figure 3). The problems are compounded by the type of reactions used in the quantification processes. To illustrate this point, let us consider the determination of total ellagitannins with the sodium nitrite method. This method relies on the hydrolysis of ellagitannins and on the reaction of ellagic acid (the most common hydrolysis product of ellagitannins) with sodium nitrite to yield a nitrosylated chromophore that is subsequently quantified by a spectrophotometer. The production of ellagic acid requires the presence of hexahydroxydiphenoyl (HHDP) group(s) in the hydrolyzed ellagitannins. Unfortunately, HHDP groups are not found in the structures of all ellagitannins, and, even when they are, they may be just a small part of a larger ellagitannin molecule. This may lead us to underestimate the ellagitannin content of plant samples, or—in extreme cases—even to overlook the mere presence of ellagitannins. In *Q. robur*, we found the leaves to contain substantial amounts of **5**, **7**, **8**, **9**, and **10**—the structures of which contain highly variable proportions of HHDP groups (77.6, 32.5, 32.5, 0, and 16.3%, respectively). Compound **9** is the extreme example as it contains only biosynthetically modified HHDP groups, i.e., a nonahydroxytriphenoyl and a valoneoyl group. Hence, for samples of oak leaves, the sodium nitrite method is likely to yield a highly biased estimate of total ellagitannin content. Earlier, the same pattern of underestimation was shown to be true with quantification of total galloylglucoses by the rhodanine method (Salminen, 2003) and with quantification of total phenolics by the Folin (Folin–Ciocalteau or Folin–Denis) assay (Appel et al., 2001). Moreover, the Folin assay also underestimated the total phenolic content of our oak leaves (compare Figure 2A and B).

Despite these shortcomings, total methods are still commonly used in ecological studies (e.g., McKinnon et al., 1999; Fisher et al., 2000; Inbar et al., 2001; Lill and Marquis, 2001; Abrahamson et al., 2003; Forkner et al., 2004). We realize that such methods may be useful under certain circumstances (Appel et al., 2001; Salminen, 2003), but stress their limitations in studies aiming to pinpoint the role of, e.g., hydrolyzable tannins as determinants of herbivore performance. Simply speaking, if we do not know the structures of the hydrolyzable tannins of our target species, we know neither what we are quantifying nor our measurement error. Hence, we recommend that chemically minded ecologists and ecologically minded chemists should focus their future analyses on individual tannins and establish the specific biological activities of these polyphenolic compounds through rigorous bioassays (cf. Salminen and Lempa, 2002).

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EFFECT OF NIGHTTIME TEMPERATURE ON TOMATO PLANT DEFENSIVE CHEMISTRY

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Abstract—Given that the amplitude of diurnal temperature fluctuations has been decreasing, mainly via warmer night temperatures, we examined the effects of nighttime temperature on concentration of the catecholic phenolics chlorogenic acid and rutin in tomato plants. A two-factor design, with carbon dioxide (350 ppm and 700 ppm) and nighttime temperature (14, 15, 16, 17, and 18°C, with a 26°C daytime temperature) was used. Compared to the lower carbon dioxide level, for whole plants the concentration of phenolics was lower at the higher carbon dioxide level, but patterns for plant parts differed. Nighttime temperature did not affect concentration of phenolics for whole plants, but it did influence concentration of the phenolics for plant parts, although not in predictable ways. Furthermore, the pattern of concentration of chlorogenic acid was somewhat different from that of rutin. The amount of change in concentration of these allelochemicals is likely sufficient to have substantial effects on insect herbivores. We conclude that nighttime temperature affects concentration of allelochemicals in tomato plants in significant ways.

Key Words—Carbon dioxide, chlorogenic acid, *Lycopersicon esculentum*, plant defense, rutin.

INTRODUCTION

One effect of global warming in the last 40 years is a decrease in the amplitude of diurnal temperature fluctuations, mainly via warmer night temperatures (Idso and Balling, 1991; Karl et al., 1991, 1993). Data over the past four decades show that the mean minimum (mostly nighttime) temperature rose while the mean maximum (mostly daytime) temperature remained relatively unchanged (Karl et al., 1991, 1993). The monthly mean minimum temperature has risen at a rate three times that of maximum temperature (0.84°C vs. 0.28°C, respectively) (Karl et al., 1991,

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1993). Changes in cloudiness (sky cover and cloud ceiling height) explain the greatest proportion of the variance in diurnal temperature amplitude (Karl et al., 1993). In addition, the increase in nighttime temperature was most striking during the growing season.

Most plant growth occurs at night (Went and Bonner, 1943; Went, 1944), and many plants grow better with nighttime temperature several °C below daytime temperature (Went, 1944; Dorland and Went, 1947; Friend and Helson, 1976; Ivory and Whiteman, 1978). With cooler nighttime temperature, less of the daily gain in fixed carbon may be expended on respiration (Hewitt and Curtis, 1948; Hogan et al., 1991). Therefore, more fixed carbon may be available for growth and defense.

One factor contributing to the current global warming is the increase in carbon dioxide due to industrialization (IPCC, 1996). Experiments contrasting the effects of ambient and elevated (usually doubled) CO_2 on plants have shown that, with CO_2 enrichment, non-nitrogenous defenses in leaves increase, although not for all species (Lincoln et al., 1986; Lincoln and Couvet, 1989; Fajer et al., 1991; Cipollini et al., 1993; Julkunen-Tiitto et al., 1993; Lindroth et al., 1993).

In this study, the effect of climate change via warmer nights on defense of plants was examined by manipulating both CO_2 and nighttime temperature.

METHODS AND MATERIALS

The plant species was tomato (*Lycopersicon esculentum*: Solanaceae). Previous studies showed that tomato plants had the highest growth rate when daytime temperature was about 26°C and nighttime temperature was about 13–18°C (Went, 1944, 1945). Within the normal thermal range, nighttime temperature had a greater effect on growth rate than daylight intensity, length of daylight period, and relative humidity (Went, 1944). Rutin and chlorogenic acid are the major phenolics in tomato and occur widely in terrestrial plants (Sondheimer, 1964; Harborne, 1979; Isman and Duffey, 1982). The concentrations of rutin in tomato leaves range up to 20 μmoles/g fresh weight, with an average of 4 μmoles (Duffey et al., 1986); while the concentrations of chlorogenic acid in tomato foliage range up to 20 μmoles/g fresh weight, with an average of 13 μmoles (Elliger et al., 1981; Duffey et al., 1986). These allelochemicals can have negative effects on insect growth (Elliger et al., 1981; Isman and Duffey, 1982; Stamp and Yang, 1996).

Seeds (var. Heinz) were planted in flats containing soil with time-release fertilizer (Agway Professional Potting Mix, 0.09% nitrogen, 0.09% phosphate, and 0.09% potash) and placed in assigned plant growth chambers, with a photon flux density of 850 μmoles/m^2/sec with a 14L:10D photocycle. Two weeks after planting (which was a week after emergence), seedlings were transplanted individually into 750 ml (13-cm diam) pots. Plants were watered as necessary, and the chambers had pans of water that maintained relative humidity at about 50%.

A factorial design was used, with one factor as nighttime temperature and the other as carbon dioxide. The thermal regime was a daytime temperature of 26°C, with a nighttime temperature of 14, 15, 16, 17, or 18°C. Separate trials ensured that each chamber was assigned once to each nighttime temperature. The daytime temperature of 26°C was used because it is the optimal daytime temperature for tomato growth (Went, 1944, 1945). To examine the effect of changes in nighttime temperature, the daytime temperature was held constant, even though doubling of CO_2 influences daytime temperature, too, but to a much lesser extent than nighttime temperature. The nighttime temperatures were based on the prediction of 3°C (±2) increase in global temperature with a doubling of CO_2 (IPCC, 1990), and data showing that the change in daily average maximal:minimal (daytime:nighttime) temperatures is 1:3 in temperate North America (Karl et al., 1991).

To determine the effects of elevated CO_2, two levels were contrasted: 350 ppm and 700 ppm. The latter value is the average of model predictions for ambient concentration by 2100 (IPCC, 1996). Chamber CO_2 concentration was maintained within 5% of the set-point, with an infrared-gas analyzer and controller unit.

Three weeks after transplanting, leaves and stems were harvested, and roots were washed free of soil. Plant parts were oven-dried (55°C). A sample of stems, roots, and intermediate-sized leaflets were ground to powder for chemical analysis.

Concentrations of chlorogenic acid and rutin were determined by standard procedures (Broadway et al., 1986; Wilkens et al., 1996). Phenolics were extracted in methanol. Diphenylboric acid, a dye that is specific for catecholic phenolics, was used. Because chlorogenic acid and rutin have overlapping absorbencies, the concentrations in leaf samples were calculated using difference equations based on the extinction coefficients of the two phenolics (Broadway et al., 1986). Commercial preparations of chlorogenic acid and rutin (from Sigma Chemical) were used to develop standard curves.

Two-way ANOVA was conducted with the factor of CO_2 (350 and 700 ppm) and the factor of nighttime temperature (5 levels). There were 30 replicates for the chemical analyses of plant parts and 10 replicates for whole plant analysis, except in a few instances when part of a sample was incomplete.

RESULTS

Compared to the lower CO_2 level, concentration of catecholic phenolics (chlorogenic acid and rutin) for whole plants was reduced at the higher CO_2 level ($F_{1,90} = 4.23$, $P = 0.04$), but nighttime temperature did not have an effect ($F_{4,90} = 0.21$, $P = 0.93$).

However, within plant parts, there were different patterns. The concentration of catecholic phenolics in leaves was greater at the higher CO_2 level, higher at 17°C than the other nighttime temperatures, and the patterns across the nighttime temperatures at the two CO_2 levels were dissimilar (Table 1, Figure 1). The con-

TABLE 1. EFFECT OF CARBON DIOXIDE AND NIGHTTIME TEMPERATURE ON LEAF, STEM, AND ROOT CONCENTRATIONS OF CATECHOLIC PHENOLICS[a]

	df	*F*	*P*
Leaf			
CO_2	1,282	4.192	0.04*
Night temperature	4,282	10.961	<0.001*
CO_2 × Temperature	4,282	3.324	0.01*
Stem			
CO_2	1,269	27.073	<0.001*
Night temperature	4,269	3.957	0.004*
CO_2 × Temperature	4,269	2.091	0.08
Root			
CO_2	1,246	3.749	0.06
Night temperature	4,246	2.406	0.05*
CO_2 × Temperature	4,246	2.641	0.03*

[a] ANOVA with asterisk (*) indicating $P < 0.05$ at $\alpha = 0.05$.

centration of phenolics in stems was lower at the higher CO_2 level and lower at 18°C than the other nighttime temperatures (Table 1, Figure 1). The concentration of phenolics in roots was not affected by CO_2 level *per se*, but declined with increasing nighttime temperature, and the patterns across the nighttime temperatures at the two CO_2 levels were dissimilar (Table 1, Figure 1).

The pattern for chlorogenic acid showed no effect of CO_2 in leaves, but for stems and roots, there was reduced concentration at the higher CO_2 level (Table 2, Figure 2). Nighttime temperature had an effect on chlorogenic acid concentration. With an increase in nighttime temperature, there was an increase in concentration in leaves. For stems, there was a curvilinear response peaking at 17°C and, for roots, a difference in response to CO_2 levels at 15 and 16°C (Table 2, Figure 2).

The pattern for rutin showed an increase in concentration in leaves and roots at the higher CO_2 level, but a decrease for stems (Table 2, Figure 2). Nighttime temperature had an effect on rutin concentration in leaves and stems but not in roots (Table 2). For leaves, rutin concentration was greater at 17°C than at the other nighttime temperatures (Figure 2). For stems, the effect of nighttime temperature on rutin concentration was a function of CO_2 level, with a relatively low concentration at 18°C with the higher CO_2 level and relatively high concentration at 15°C with the lower CO_2 level (Figure 2).

DISCUSSION

That CO_2 level affected leaf concentration of these catecholic phenolics was expected. For example, with CO_2 doubled from ambient, tobacco leaves

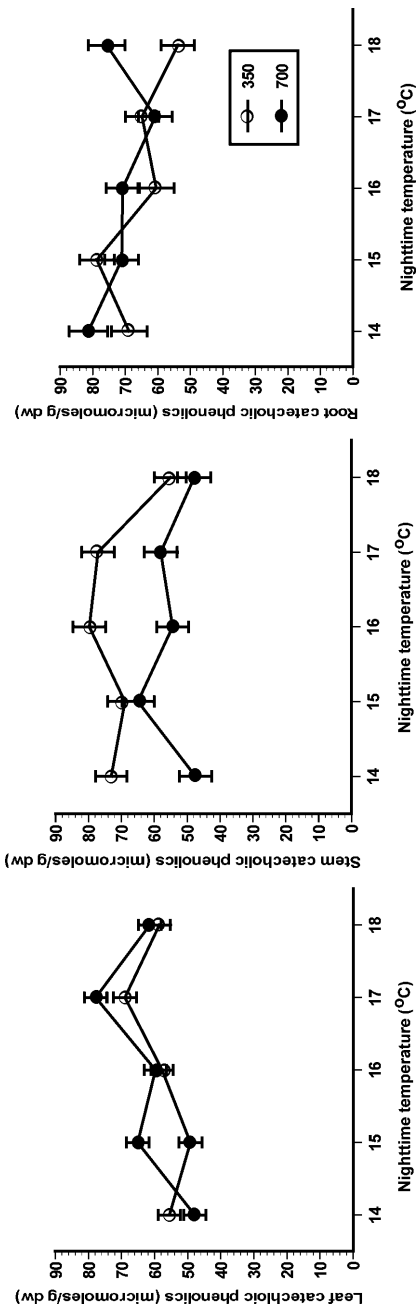

FIG. 1. Effect of carbon dioxide (350 ppm vs. 700 ppm) and nighttime temperature (14, 15, 16, 17, and 18°C) on concentration of catecholic phenolics in tomato leaves, stems, and roots.

TABLE 2. EFFECT OF CARBON DIOXIDE AND NIGHTTIME TEMPERATURE ON CONCENTRATIONS OF CHLOROGENIC ACID AND RUTIN IN PLANT PARTS[a]

	Chlorogenic acid			Rutin		
	df	*F*	*P*	df	*F*	*P*
Leaf						
CO_2	1,282	0.033	0.85	1,282	5.789	0.02*
Night temperature	4,282	6.216	<0.001*	4,282	6.947	<0.001*
$CO_2 \times$ Temperature	4,282	2.997	0.02*	4,282	1.566	0.18
Stem						
CO_2	1,269	9.808	0.002*	1,269	21.185	0.001*
Night temperature	4,269	9.652	0.001*	4,269	2.844	0.03*
$CO_2 \times$ Temperature	4,269	1.234	0.30	4,269	3.273	0.01*
Root						
CO_2	1,246	5.878	0.02*	1,246	7.734	0.006*
Night temperature	4,246	4.021	0.003*	4,246	2.120	0.08
$CO_2 \times$ Temperature	4,246	3.219	0.01*	4,246	2.357	0.06

[a] ANOVA with asterisk (*) indicating $P < 0.05$ at $\alpha = 0.05$.

had higher concentrations of the phenolics chlorogenic acid and rutin (Rufty et al., 1989). The general explanation for an increase in concentration of non-nitrogenous defenses is that there is a dilution effect, due to greater availability of carbon without a corresponding increase in nitrogen. In our study, plants were provided with fertilizer (as is typical of crops), so defenses requiring nitrogen in their production (and all defenses require some nitrogen) are less likely to decline with CO_2 enrichment through a dilution effect. At the whole plant level, however, concentration of catecholic phenolics was *lower* at the higher CO_2 level, even though concentration of these chemicals in leaves was higher at the higher CO_2 level than at the lower CO_2 level. This contrast reflects that the bulk of plant dry weight is in the non-leaf parts, in particular the stems, where phenolic concentration was less at the higher CO_2 level.

What are the implications for effects on insect herbivores? The changes in concentrations exhibited in this study, for example, $10 \pm \mu$moles/g dry weight, are sufficient to reduce weight gain and/or developmental rate of insects substantially (Yang and Stamp, 1995; Stamp and Yang, 1996; Stamp and Osier, 1997, 1998). In addition, studies on the simultaneous effects of temperature and food quality suggest that temperature can also affect insect performance by altering the effects of food quality (Schramm, 1972; Stamp, 1990; Stamp and Bowers, 1990; Salim and Saxena, 1991; Stamp et al., 1991; Yang and Joern, 1994; Stamp and Yang, 1996). Overall, these results suggest that there may be shifts in the susceptibility of insects to allelochemicals as nighttime temperature increases with global warming. Therefore, to assess the ecological consequences of temperature and specifically changes in nighttime temperature due to global warming, it is imperative to collect

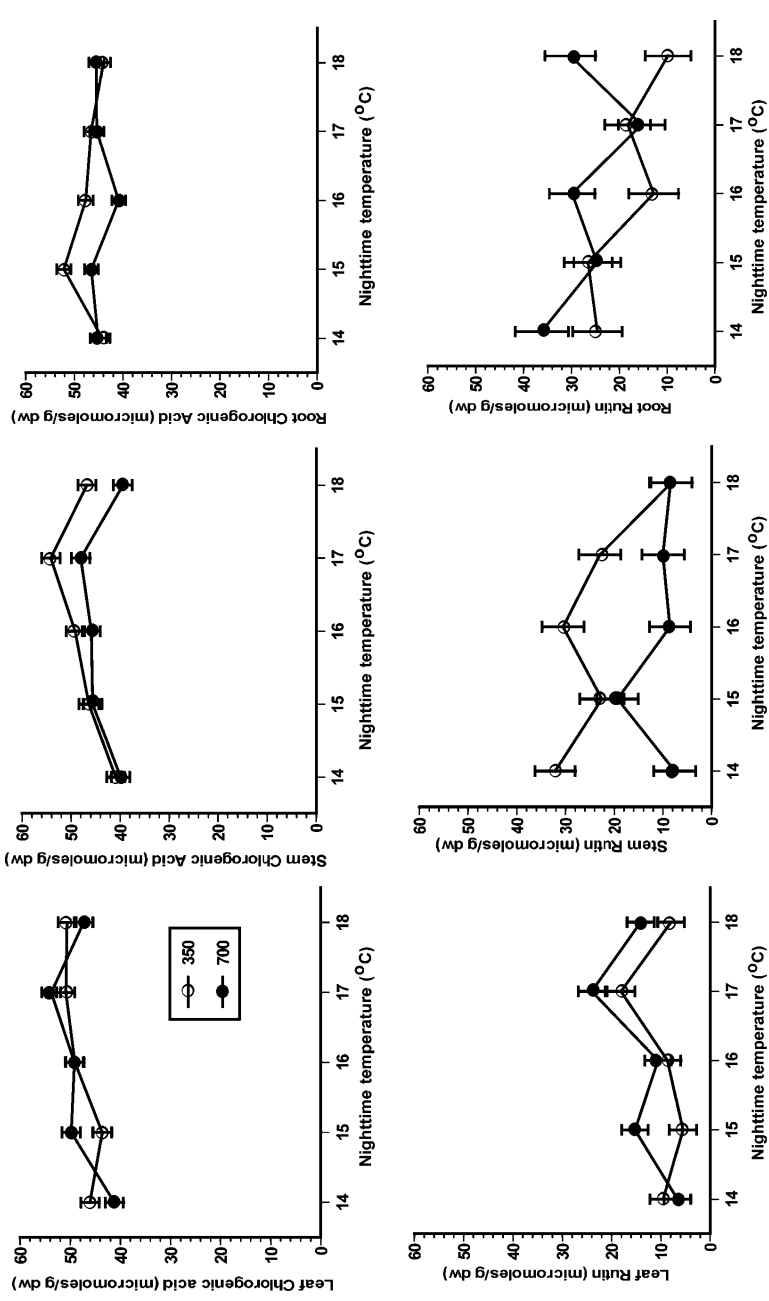

FIG. 2. Effect of carbon dioxide (350 ppm vs. 700 ppm) and nighttime temperature (14, 15, 16, 17, and 18°C) on concentration of chlorogenic acid and rutin in tomato leaves, stems, and roots.

data on the growth and phytochemistry of plants of various stages (e.g., seedling vs. adult) and, in turn, on growth of insect herbivores under different nighttime temperatures.

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PATTERNS OF IRIDOID GLYCOSIDE PRODUCTION AND INDUCTION IN *Plantago lanceolata* AND THE IMPORTANCE OF PLANT AGE

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Abstract—Induction of allelochemicals is one way that plants efficiently deploy defenses against herbivory. In two separate experiments we investigated the time course of this inductive response and the importance of the timing of herbivory for *Plantago lanceolata* (Plantaginaceae). We found a localized induced response of catalpol and the ratio of catalpol to total iridoid glycosides in damaged leaves that was evident at d 6 after caterpillars of the specialist *Junonia coenia* were put onto the plants. On the whole plant level, we detected small, but significant changes in the iridoid glycoside metabolism of *P. lanceolata* on several different days following herbivory. We also found considerable change in the amounts of allelochemicals produced during *P. lanceolata's* ontogeny. This ontogenetic effect might help to explain some of the reasons why induction may be difficult to detect in *P. lanceolata*. We also investigated the importance of the timing of herbivory on *P. lanceolata*'s inductive response, but neither herbivory after 5 wk of growth nor after 6 or 7 wk of growth induced an increase in aucubin or catalpol.

Key Words—*Junonia coenia*, aucubin, catalpol, induced response, plantain, Plantaginaceae.

INTRODUCTION

Plants contain a wide diversity of allelochemicals. These may serve a diversity of functions, including direct defense against herbivores and pathogens, indirect defense by serving as attractants for herbivore predators and parasitoids, screens against ultraviolet light, and protection against water loss. Constitutive levels of

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these compounds vary among species, populations, individuals, and plant parts (Krischik and Denno, 1983). They may be affected by a variety of environmental factors such as light and nutrient availability (e.g., Waterman and Mole, 1989). In addition to these sources of variation, plants may change levels of allelochemicals as they grow (Zangerl et al., 1997; Hagele and Rowell-Rahier, 1999) and in response to attack by herbivores or pathogens (Karban and Baldwin, 1997; Agrawal et al., 1999; Tollrian and Harvell, 1999).

Induction of plant allelochemicals has been observed in many plant species (Karban and Baldwin, 1997; Agrawal et al., 1999; Tollrian and Harvell, 1999); however, the interaction of plant ontogeny and timing of herbivory with inductive responses has been much less studied (but see Ohnmeiss and Baldwin, 2000). These inductive responses vary from very fast, localized responses [minutes to hours, e.g., wild parsnip, *Pastinaca sativa* (Apiaceae), Zangerl and Berenbaum, 1995; wild tobacco, *Nicotiana sylvestris* (Solanaceae), Baldwin, 1987; tomato, *Lycopersicon esculentum* (Solanaceae), Stout et al., 1996], to localized or systemic responses that occur over days or weeks [e.g., *P. lanceolata* (Plantaginaceae), Darrow and Bowers, 1999], to very slow responses [months to even years, e.g., mountain birch, *Betula* (Betulaceae), Haukioja and Niemelä, 1979; Neuvonen and Haukioja, 1991)]. As a result, different plant parts may have elevated allelochemicals at certain times and not at others; or some individual plants in a population may be induced while others are not.

Narrow-leaved plantain, *Plantago lanceolata* L. (Plantaginaceae) is a cosmopolitan, annual or perennial herb that is native to Europe and was introduced to North America about 200 years ago (Cavers et al., 1980; Thomas et al., 1987). It has been incorporated into the diets of many native North American insect species (e.g., Thomas et al., 1987) and is commonly used as a hostplant by the insect herbivore used in the present study, the Buckeye butterfly, *Junonia coenia* Hübner (Nymphalidae: Lepidoptera) (Bowers, 1984; Scott, 1986). *P. lanceolata* has been widely studied in Europe, but less so in North America (Kuiper and Bos, 1992). The plant produces primarily the iridoid glycosides aucubin and catalpol (Bowers et al., 1992), but also smaller amounts of two other iridoid glycosides, 8-epiloganic acid and gardoside (Willinger and Dobler, 2001). Aucubin and catalpol serve as feeding or oviposition stimulants for some specialist insect species, are deterrent or toxic to some generalist insect species, and are sequestered by certain insect species and used as a defense against their own predators (reviewed in Bowers, 1991).

Genetic and environmental factors affect iridoid glycoside production in *P. lanceolata* (Fajer et al., 1991, 1992; Bowers et al., 1992; Bowers and Stamp, 1993; Darrow and Bowers, 1999; Jarzomski et al., 1999; Marak et al., 2002b). Although some experiments with herbivores have shown induction of iridoid glycosides (e.g., Bowers and Stamp, 1993; Darrow and Bowers, 1999), others have not (e.g., Stamp and Bowers, 1996; Jarzomski et al., 1999).

We used *P. lanceolata* as a model organism to address three questions about iridoid glycoside production and induction: (1) How does iridoid glycoside production change over the development of the plant; (2) Is iridoid glycoside induction a localized or a systemic phenomenon and how does this induction proceed; (3) How does the timing of herbivory affect induction of iridoid glycosides?

METHODS AND MATERIALS

Study Organisms. Seeds were collected from a *P. lanceolata* population in Davis, CA, and germinated in shallow plastic trays filled with Fafard potting soil (Canadian Sphagnum Peat Moss 70%, Perlite 15%, Vermiculite 15%). Nine days after seeds germinated, seedlings were transplanted into 1-gallon plastic pots (diam = 15 cm). Transplanted seedlings were watered once with nutrient-enriched water containing 164-ppm nitrogen, 33.5-ppm magnesium, 50-ppm calcium, and low amounts of the trace elements—boron, copper, zinc, iron, manganese, and molybdenum. Plants were grown under natural light conditions in the greenhouse throughout the experiment. Greenhouse conditions were as follows: 30% humidity, a heater that came on at 13°C and a cooler that came on at 24°C, the vents opened for passive cooling at 22°C. Each plant was watered every 3 d with 500 ml of tap water and was never allowed to dry out. Each experiment was carried out in three blocks, and plants were shuffled weekly within blocks.

J. coenia is a new world butterfly that produces one to three broods per year in the wild and uses various members of the Scrophulariaceae, Verbenaceae, Cornaceae, and Plantaginaceae as host plants (Bowers, 1984; Scott, 1986). All of the documented hostplant species produce iridoid glycosides (IG) (Bowers, 1984). Larvae can accumulate up to 25% dry weight in IG depending on the IG content of the plant (Camara, 1997; Theodoratus and Bowers, 1999). *J. coenia* caterpillars used in the experiments came from a colony reared at the University of Colorado that was fed on *P. lanceolata* leaves or (rarely) an artificial diet containing dried *P. lanceolata* powder. Larvae were kept in growth chambers under conditions of 14 hr, 28°C, in day and 10 hr, 22°C, in night.

To confine the caterpillars on the plants during the herbivory treatments, plants were caged with mesh bags. Control plants were caged in the same way and for the same time period as the treatment plants.

Experiment 1—Timing of Induced Responses. Seeds were started on January 1, 1999, and transplanted on January 19, 1999. From a total of 218 plants, 116 were randomly assigned to the control treatment (=no herbivory) and 102 to the treatment with *J. coenia* caterpillars (=herbivory). On February 24, the herbivory treatment began and two *J. coenia* caterpillars, one 4th and one 5th instar were placed into the center of the treatment plants, and all plants (both experimental and control) were caged. At the same time, the above-ground portions of 10 control plants were harvested. On February 27, caterpillars and cages were

removed from the plants. The amount of herbivory on each treatment plant was estimated at this time (see below).

On d 3, 4, 6, 8, 10, 13, 17, 24, and 27 after the beginning of the treatment, above-ground parts of a set of control and treatment plants (9–10 plants each) were harvested. All plants were cleaned of soil, and the leaves of the treatment plants were separated into those that had been chewed upon by the caterpillars ("chewed") and those that showed no sign of herbivory ("unchewed"). Leaves from control plants and chewed and unchewed leaves from the treatment plants were then placed into separate, labeled paper bags, dried at 50°C, and then weighed.

The amount of herbivory inflicted was determined by taking a picture of each plant with a Kodak DC120 Zoom Digital Camera immediately before and then immediately after the treatment. Images were enhanced with Adobe Photoshop if necessary and printed on an Epson Stylus Pro Esc/P2 color laser printer. Using these pictures, the amount of damage was estimated to the nearest 5%.

Experiment 2—Induction and Timing of Herbivory. A second cohort of seeds was planted on January 25, 1999, and transplanted on February 2, 1999. Ninety plants were randomly assigned to one of three groups (30 plants per group): (1) herbivory at 5 wk, (2) herbivory at 6 wk, and (3) herbivory at 7 wk. Within each of these groups, there were three subgroups: (a) plants exposed to herbivores, (b) 10 "beginning control" plants harvested at the same time that the caterpillars were placed onto the treatment plants, and (c) 10 "end-control" plants harvested at the same time that the treatment plants were harvested. Group 1 was treated with three *J. coenia* caterpillars per plant 5 wk after transplanting (March 9). Group 2 and 3 plants (wk 6 and 7, respectively) were subject to herbivory by five caterpillars per plant. In order to try and inflict the same proportion of damage on each plant, five caterpillars instead of three were used in Groups 2 and 3, because plants had more leaves after wk 6 and 7 as compared to after wk 5. Caterpillars remained on the leaves for 2–3 d in each treatment group. The exact amount of time varied because we were trying to keep the amount of damage similar in all treatments. Both treatment plants and corresponding controls were caged during this time.

Beginning controls were harvested when caterpillars were put on the treatment plants (group 1, March 9; group 2, March 16; group 3, March 23). End controls and treatment plants were harvested 10 d after the beginning of the treatment in each group (wk 5, March 19; wk 6, March 26; wk 7, April 2). Plants were harvested on d 10 following the treatment because results from the first experiment indicated that we would be most likely to detect an induced response at this time. Harvested plants were cleaned and put into labeled paper bags, dried at 50°C to a constant weight, and weighed.

Chemical Analysis. Plants were ground to a fine powder and 25–35 mg from each plant were used for quantification of IG by gas chromatography. Samples were extracted overnight in 5 ml of methanol, filtered of solid particles the following day, and evaporated to dryness. An internal standard [phenyl-β-D-glucose

(PBG)] was added, and the sample was partitioned between water and ether to remove hydrophobic compounds and chlorophylls. The aqueous solution, containing primarily IG and sugars, was evaporated to dryness, and the residue was resolubilized in 1 ml of methanol over night. An aliquot of 100 μl was then removed, evaporated, and the residue derivatized with Tri-Sil Z (Pierce Chemical Company, Rockford, IL). After derivatizing, samples were injected into an Hewlett-Packard 5890A GC equipped with an Hewlett-Packard 3393A integrator (see also: Gardner and Stermitz, 1988).

Statistical Methods. In all experiments, the SPSS statistical package was used to perform statistical analysis, and data were arcsine square-root transformed before analysis to normalize distributions. In experiment 1, for both the comparison of treatment plants *vs.* controls and chewed *vs.* unchewed leaves, no bench effect could be detected, and, thus, bench was not considered as an independent variable in all further analyses. Two-way ANOVA was used to test for the effect of treatment, harvest date, and the interaction of treatment and harvest date on treatment plants and controls, and independent *t* tests were performed to additionally test for significant differences between treatment and control plants at the individual harvest dates. Repeated measure ANOVA was used to compare chewed *vs.* unchewed leaves on treatment plants. For all *t* tests, *P* values were subject to a sequential Bonferroni analysis (Rice, 1989) to adjust for simultaneous tests.

In experiment 2, ANOVAs were conducted first with timing of herbivory and treatment as independent variables with the whole data set. In addition we performed *post hoc* contrasts to test the means of "beginning control" *vs.* "end control" plants (time effect) and "end control" to "treatment" plants (herbivory effect). Then each treatment group (wk 5, 6, and 7) was analyzed separately by using ANOVAs and the same contrasts with treatment as the only independent variable.

RESULTS

Timing of Induced Responses (Experiment 1). Estimates from digital camera images showed that an average leaf area of 14.2% ($\pm$2.4 SE) was removed by *J. coenia*.

Chewed vs. Unchewed Leaves on Plants Exposed to Herbivores. To compare biomass and IG content of chewed and unchewed leaves from plants exposed to herbivores, we used a repeated measures ANOVA, with chewed and unchewed leaves as the within-subjects effect, and time as the between-subjects effect. Leaf biomass changed significantly over time, and there was a significant difference between the biomass of chewed and unchewed leaves. The increase in biomass of chewed leaves was probably due to leaf expansion. In addition we found a significant interaction between time and herbivory, which either indicates that

chewed and unchewed leaves reacted differently over time, or this is simply an effect of new leaves that were formed after the herbivores had been removed from the plants (Table 1, Figure 1). Paired *t* tests showed that the change in biomass between chewed and unchewed leaves was only significant at d 24 and 27, when the biomass of the unchewed leaves began to increase dramatically (Figure 1). Previous to that, biomass of chewed and unchewed leaves was similar (for all *t* tests, $P > 0.05$, Figure 1).

A comparison of the IG concentrations of the chewed and unchewed leaves showed that time significantly affected IG concentrations and the proportion of the total iridoid glycosides (TIG) that was catalpol (referred to hereafter as the proportion catalpol) (Table 1). Our analysis showed that, overall, the chewed and unchewed leaves also differed significantly in aucubin, catalpol, TIG, and the proportion of catalpol (Table 1). Paired *t* tests showed that chewed leaves had higher concentrations of aucubin, catalpol, and TIG, and a higher proportion catalpol on d 6 only (Figure 2). Although there are other days where it appears that there were differences between chewed and unchewed leaves (e.g., proportion catalpol on d 4), these were not significant after Bonferroni corrections were applied.

In addition, the proportion catalpol increased in the chewed leaves and decreased in the unchewed leaves between d 0 (when herbivory started) and d 3 when herbivory ended. Interestingly, aucubin and TIG declined in both unchewed and chewed leaves from d 0 when caterpillars were added to the plants and appeared to be higher in unchewed leaves on d 3, but this was not significant after Bonferroni correction. On d 8, 10, 13, and 17, there were no differences in any of the IG measures of the unchewed and chewed leaves (*t* tests > 0.05, Figure 2). By d 24, unchewed leaves were higher in aucubin and TIG, although there was no change in catalpol and the proportion catalpol (*t* tests >0.05, Figure 2).

The interaction of time and treatment was significant for all measures of IG concentrations (Table 1). This means that the effect of herbivory on iridoid glycoside concentrations in chewed *vs*. unchewed leaves was different over time. This effect was probably due to the drop in aucubin, catalpol, and TIG concentrations in the chewed leaves on d 24 and 27 (Figure 2).

Plants Exposed to Herbivores vs. Control Plants. We calculated the concentration of IG at a whole plant level in the plants exposed to herbivores by correcting for the biomass of the chewed and unchewed leaves. Then we compared these concentrations for plants exposed to herbivores with the control plants. We used a two-way ANOVA, with time and treatment (control or exposed to herbivores) as the main effects, to compare the control and treatment plants.

Time significantly affected plant biomass (Table 2, Figure 3); however, there was no main effect of treatment on biomass (Table 2). There was a significant

TABLE 1. REPEATED MEASURES ANOVA ON THE BIOMASS AND IRIDOID GLYCOSIDE CONCENTRATIONS OF CHEWED AND UNCHEWED LEAVES OF PLANTS EXPOSED TO HERBIVORES IN EXPERIMENT 1

	Between-subjects effect = Time			Within-subjects effect = Chewed vs. unchewed			Time × Treatment			Error
Vaiable	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df
Biomass	1	137.907	<0.001***	1	13.360	<0.001***	1	65.859	<0.001***	86
Aucubin	1	32.374	<0.001***	1	15.663	<0.001***	1	55.917	<0.001***	86
Catalpol	1	56.901	<0.001***	1	17.057	<0.001***	1	23.104	<0.001***	86
Total iridoid glycosides	1	43.796	<0.001***	1	17.708	<0.001***	1	58.028	<0.001***	86
Proportion catalpol	1	36.967	<0.001***	1	19.506	<0.001***	1	5.896	<0.017*	86

* $P < 0.05$; *** $P < 0.001$.

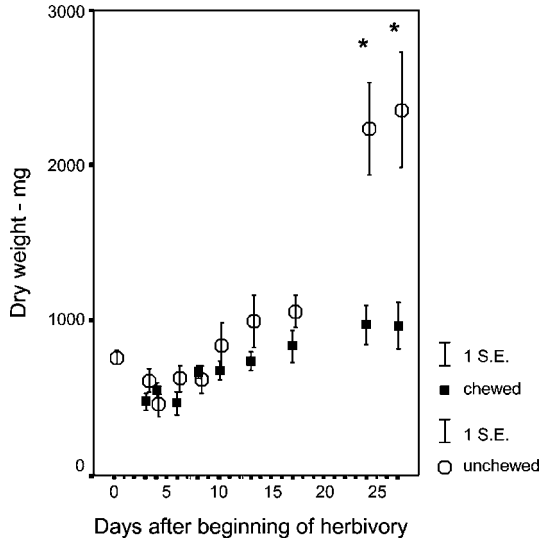

FIG. 1. Changes in dry weight of chewed and unchewed leaves in *Plantago lanceolata* over the 27 d of the experiment. Asterisks (*) denote significant results in paired t tests. Means $\pm$ one standard error are shown.

time by treatment interaction (Table 2), because, although until d 17 control plants were higher in biomass than plants exposed to herbivores, this changed on d 24 as the herbivory plants recovered. t-Tests showed that there were no significant differences in the biomass of control plants and those exposed to herbivores on any individual day, except for d 17 ($t = 2.753$, df $= 18$, $P = 0.013$) (Figure 3).

All measures of IG concentrations were significantly affected by time (Table 2, Figure 4). The concentrations of IG in both control plants and plants exposed to herbivores increased from less than 0.5% to over 6% dry weight. Thus, in only 28 d, the plants dramatically increased their production of IG.

The presence *vs*. absence of herbivores only significantly affected catalpol concentration and the proportion catalpol (Table 2, Figure 4). t-Tests showed that on d 6 and 10 the catalpol concentration was higher in plants exposed to herbivory ($P < 0.05$) and that on d 4, 6, 10, and 27 the proportion catalpol was significantly higher in plants exposed to herbivores ($P < 0.05$). As in the comparison of chewed and unchewed leaves, on some days there appeared to be differences in IG between plants exposed to herbivores and the control plants (e.g., aucubin and TIG on d 10 and catalpol on d 17), but these were not significant after Bonferroni corrections were applied.

Both treatment and control plants showed a considerable increase in the amount of catalpol and the ratio of catalpol to TIG on d 13 (Figure 4B and D).

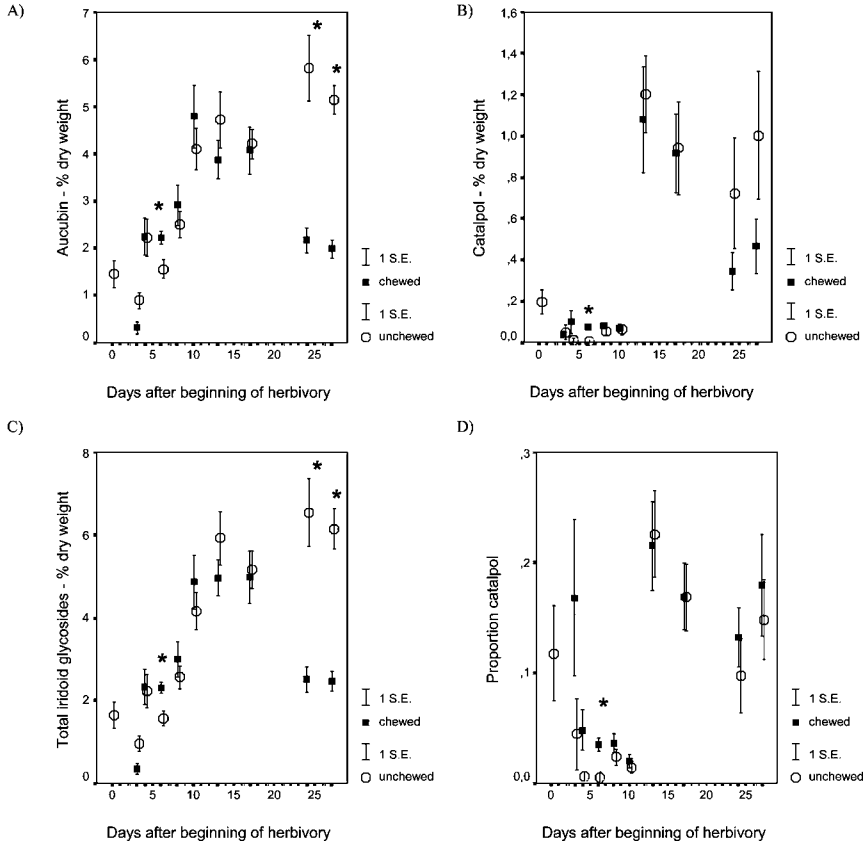

FIG. 2. Changes in iridoid glycoside concentration in chewed and unchewed leaves in *Plantago lanceolata* over the 27 d of the experiment. A. Aucubin concentration. B. Catalpol concentration. C. Total iridoid glycoside concentration. D. Catalpol/total iridoid glycosides. Asterisks (*) denote significant results in paired *t* tests. Means ± one standard error are shown.

There was no such increase for aucubin or TIG, although both increased as plants grew over the 27 d of the experiment (Figure 4).

Induction and Timing of Herbivory (Experiment 2). Digital camera images indicated that leaf-area removal was approximately as follows: wk 5 = 9% (±1.6 SE); wk 6 = 17.5% (±2.9 SE); wk 7 = 17.5% (±3.8 SE).

ANOVA revealed several features of the pattern of IG production in this experiment. (1) Concentrations of aucubin and TIG were significantly affected by treatment, however, this effect was not due to herbivory (see below and contrasts Table 3). (2) Concentrations of aucubin, catalpol, and TIG, but not the

TABLE 2. TWO-WAY ANOVA ON BIOMASS AND IRIDOID GLYCOSIDE MEASURES FROM EXPERIMENT 1, COMPARING PLANTS EXPOSED TO HERBIVORES WITH CONTROL PLANTS[a]

	Time			Treatment = Herbivory vs. Control			Time × Treatment			Error
Vaiable	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df
Biomass	9	34.586	<0.001***	1	0.816	0.386	8	2.355	0.020*	166
Aucubin	9	32.486	<0.001***	1	3.147	0.078	8	2.166	0.033*	166
Catalpol	9	49.130	<0.001***	1	19.813	<0.001***	8	1.589	0.131	166
Total iridoid glycosides	9	39.717	<0.001***	1	1.252	0.265	8	2.207	0.029*	166
Proportion catalpol	9	26.605	<0.001***	1	43.961	<0.001***	8	1.921	0.060	166

[a] aBench effect was not significant in any of the analyses ($P > 0.05$) and so was deleted from the analysis to conserve degree of freedom.

* $P < 0.05$; *** $P < 0.001$.

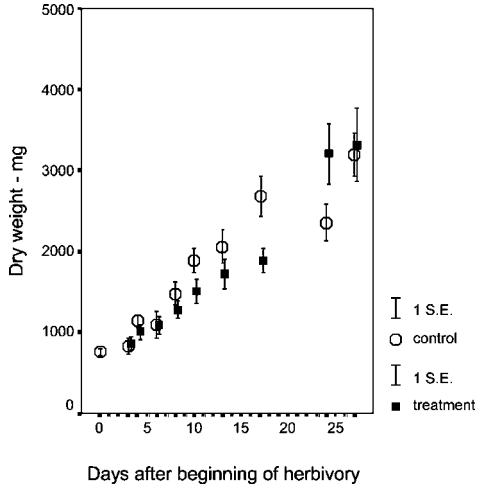

FIG. 3. Changes in dry weight of *Plantago lanceolata* exposed to herbivores over the 27 d of the experiment. Means ± one standard error are shown.

proportion catalpol were significantly affected by the time at which caterpillars were added to the plants (5, 6, or 7 wk). (3) There were no significant interactions (Table 3).

However, when the three different sampling dates (wk 5, 6, and 7) were analyzed separately, a significant effect of treatment was detected only for plants on which caterpillars were added at wk 5, and that only for aucubin and TIG ($P < 0.01$) (Figure 5). The *post hoc* contrasts showed that this effect stems from differences between "beginning control" and "end control" plants (aucubin) rather than from any effect of herbivory itself (Figure 5). Thus, we found no evidence of induction in this experiment.

DISCUSSION

Overall, our experiments showed three major results: (1) Concentration of IG in *P. lanceolata* showed an ontogenetic increase of almost an order of magnitude in only 28 d in both control plants and plants exposed to herbivores. (2) Increases in aucubin, catalpol, TIG, and the proportion catalpol in damaged leaves on plants exposed to herbivores were detected at d 6 only. At the whole plant level, on certain days catalpol and the proportion catalpol were slightly, but significantly, higher on plants exposed to herbivores. (3) The effect of timing of herbivory (when caterpillars fed on the plants) on induction could not be elucidated because we did not detect induction in that experiment.

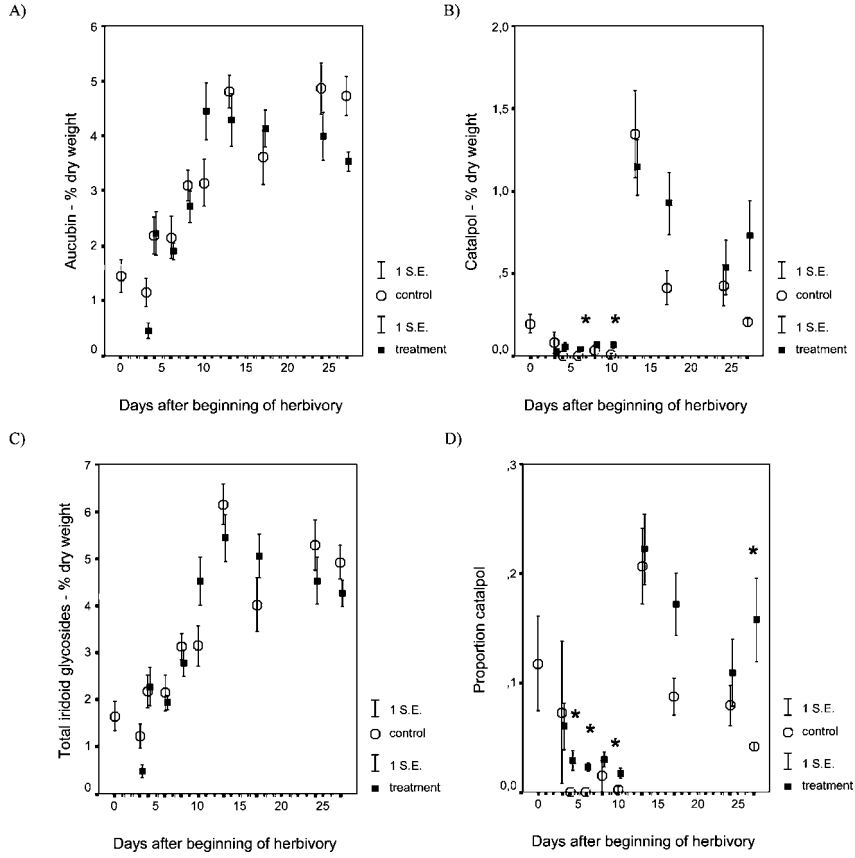

FIG. 4. Changes in iridoid glycoside concentration in control plants and plants exposed to herbivores in *Plantago lanceolata* over the 27 d of the experiment. A. Aucubin concentration. B. Catalpol concentration. C. Total iridoid glycoside concentration. D. Catalpol/total iridoid glycosides. Asterisks (*) denote significant results in independent *t* tests. Means ± one standard error are shown.

Ontogenetic Changes in Iridoid Glycosides. Concentration of IG increased over almost an order of magnitude from less than 0.5% dry weight at the start of the experiment (when plants were 5 wk after transplanting and 7 wk after seed germination) to over 6% dry weight 4 wk later (Figure 4A–D, control plants). No plants flowered during this period, so changing allocation patterns due to inflorescence initiation and development, which often occur (e.g., Zangerl et al., 1997; Høgedal and Mølgaard, 2000), were not an issue here. Thus, palatability of *P. lanceolata* may change dramatically in a relatively short period of time: for generalists, for which IG are deterrent or toxic, older plants may be much less palatable; while

TABLE 3. TWO-WAY ANOVA AND OVERALL CONTRASTS ON IRIDOID GLYCOSIDE CONCENTRATIONS FROM EXPERIMENT 2[a]

	Harvest date			Treatment			Harvest × Treatment			Error	Contrast Begining control vs. End control	Contrast: End control vs. Treatment
Vaiable	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*	df	*P*	*P*
Aucubin	2	13.872	<0.001***	2	7.672	0.001***	4	1.254	0.295	81	0.002**	1.000
Catalpol	2	3.996	0.022*	2	0.396	0.692	4	0.881	0.479	81	1.000	1.000
Total iridoid glycosides	2	12.457	<0.001***	2	5.852	0.004**	4	0.877	0.482	81	0.012*	1.000
Proportion catalpol	2	2.626	0.078	2	2.168	0.121	4	1.471	0.219	81	1.000	1.000

[a]There were three harvest dates (5, 6, and 7 wk) and 3 treatments (control-start, control-end, and treatment).
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

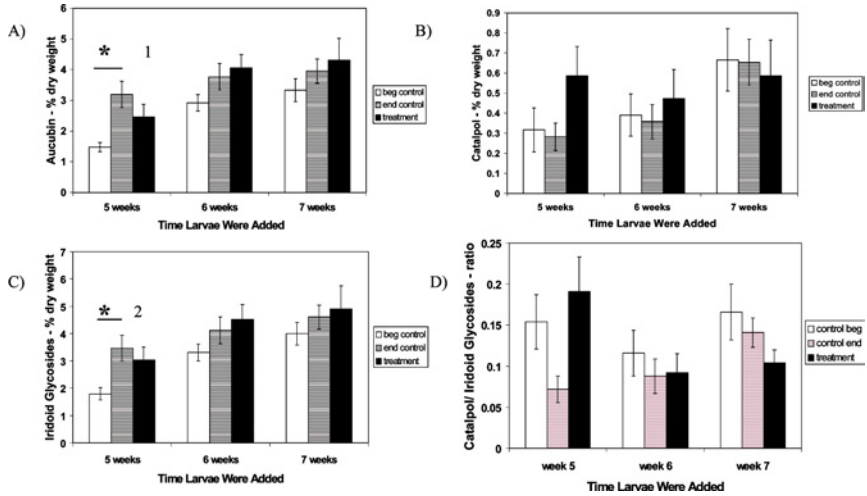

FIG. 5. Iridoid glycoside concentration in plants exposed to herbivores at different times in their ontogeny and their controls that were not exposed to herbivores. A. Aucubin concentration. B. Catalpol concentration. C. Total iridoid glycoside concentration. D. Catalpol/total iridoid glycosides. Asterisks (*) denote significant differences in the *post hoc* orthogonal contrasts (1: $P = 0.022$; 2: $P = 0.005$). Means $\pm$ one standard error are shown.

for specialists that use IG as feeding or oviposition stimulants, plants may become more attractive as they age. In addition, for insects that sequester IG, individuals feeding on older plants may be better defended than those feeding on younger plants.

Interestingly, at d 13 after the beginning of herbivory, there was an approximately 8-fold increase in catalpol and the proportion of catalpol in both plants exposed to herbivory and the control plants. Because catalpol contributed a relatively small portion of the TIG until this sampling date, this increase was quite dramatic. Since catalpol is typically the more toxic of the two IGs (Bowers and Puttick, 1988), this may result in a relatively dramatic change in the palatability of these plants to herbivores. In addition, because aucubin is the biosynthetic precursor to catalpol, this increase indicates a change in the metabolism of production of these two iridoids, with an increasing allocation to production of catalpol. The origins of this change or potential reasons for it are unknown. One possibility is the changing photoperiod, however, this increase in catalpol and the proportion catalpol occurred over only 3 d, and daylength increased by only 8 min during this time period (Boulder Colorado sunrise/sunset database).

Local and Systemic Changes in Iridoid Glycosides in Response to Herbivory. Chewed leaves showed an increase in catalpol synthesis and the proportion catalpol that was not evident until 6 d after the caterpillars were added to the plants. This

assumes that the chewed and unchewed leaves represent a random sample of leaves within plants as far as both age and position of the leaves within the plants are concerned. Looking at the damage dispersal on the digital pictures confirmed that this assumption was valid. This is important, because leaves, in the same way as the whole plants, have their own ontogeny and it is well known that leaves of different ages show different patterns of secondary chemical distribution (Krischik and Denno, 1983).

Interestingly, after 3 d, plants exposed to herbivores had lower aucubin and TIG concentrations than the control plants, but then at 10 d, plants with herbivores had higher TIG than the controls. These results indicate that IG induction in response to herbivores is quite complex in *P. lanceolata*.

The increase in catalpol, the more potent of the two IGs, in the chewed leaves (Figure 2B) may help to deter herbivores currently chewing on the leaves and in this way limit damage, especially by generalist herbivores (Puttick and Bowers, 1988). It might also help to disperse the damage (Edwards and Wratten, 1983), which may be important in how damaged plants respond to subsequent herbivory or how those plants compete for light (Croxford et al., 1989). Damage dispersal could provide the plant with a competitive advantage on the interplant level when harvesting for light, as no growing space is lost if damage is evenly distributed over the whole plant, rather than removal of an entire branch or localized section of leaves (Croxford et al., 1989). However, since we found that IG were only higher in chewed leaves on d 6, such effects would be very limited in time.

Increased production of IG may be due to increased synthesis at the points of damage, or it may be due to translocation of IG to different sites in the plant. Gowan et al. (1995) showed that another IG that is found in snap-dragons (*Antirrhinum majus*), antirrhinoside, is synthesized in leaf blades and is then translocated to buds via the phloem. A similar mechanism in *P. lanceolata* may result in translocation of IG into undamaged leaves from damaged leaves, which would explain the drastic decline in both aucubin and TIG in chewed leaves after d 17 (Figure 2B and D). This hypothesis is further corroborated by the loss of biomass and early senescence of damaged leaves (personal observation).

The pattern behind the systemic changes in IG is still more elusive. Plants that were subjected to herbivory did show higher levels of IG on some days, but these were relatively small when compared to the observed ontogenetic increases (Figure 4). However, after d 13, both catalpol and the proportion catalpol were indeed higher in plants exposed to herbivores, and significantly so on d 27 (Figure 4D). Our experiment showed that there was a change in IG metabolism in response to herbivory, but that this change was observed only for catalpol and proportion catalpol, was relatively small, and was confined to only a few days. So, the question remains, did we observe induction in response to herbivory? Karban and Baldwin (1997) define an induced response as "changes in plants following damage (or stress)." By that definition, we did observe an induced response—IG

changed after herbivory. However, this response was limited. Further experiments that explicitly take the ontogenetic effects into account and correct for this background increase are necessary to resolve this issue.

Effects of Timing of Herbivory on Induced Responses. IGs generally increased in control plants from 5 to 7 wk (Figure 5), but a significant difference in the levels of IG between "end control" and "treatment" plants, which would be an induced response, was not found. It may be the case that *P. lanceolata* is only inducible at certain ontogenetic stages. A few of the plants from the 7-wk treatment had begun initiation of flowering stalks, although these were at an early stage. It may be that all plants were already redirecting resources as they approached the time of reproduction, resulting in a lack of inducibility. A similar case was reported in wild tobacco (Baldwin and Schmelz, 1994).

In summary, induced responses in *P. lanceolata* and other plants may only be detected at certain times in a plant's ontogenetic development. Thus, experiments investigating induced responses need to take this ontogenetic component into consideration. This is especially true for species that have not been previously investigated for induced response because different species may show different patterns of induction (see Karban and Baldwin, 1997). If damage occurs at certain times during development, then there may be no detectable induced response; while at other times there may be a dramatic response. Thus, ontogenetic changes in plant allelochemistry, as well as induced changes (or lack of those changes) due to herbivore damage need to be considered in interpreting the results of such experiments.

For example, several previous experiments with *P. lanceolata* have differed in whether or not induced responses to herbivores were detected and what those responses were (e.g., Bowers and Stamp, 1993; Stamp and Bowers 1994, 1996; Darrow and Bowers, 1999; Jarzomski et al., 1999). The experiments reported here suggest that factors contributing to these varying results may include (a) the age of the plants, the environmental conditions, or the ontogenetic stage at which damage occurred, (b) the length of time after herbivory that plants were analyzed for an induced response, and (c) whether and when an induced response was looked for in damaged leaves only or at a whole plant level.

From the perspective of herbivores feeding on *P. lanceolata*, the induced response in damaged leaves that we found may be important under some circumstances and not in others. The increase of IGs as plants grew means that older plants (at least to the age at which we examined them) are likely to be better defended against generalist herbivores than younger plants. Relative to this ontogenetic increase in IG, the induced response to herbivory was relatively small. However, much of that response was due to increased biosynthesis of catalpol, and previous feeding experiments have shown that catalpol is more toxic to generalists than aucubin (Bowers and Puttick, 1989). If catalpol truly is the more potent of the two iridoids and if we assume that the conversion of aucubin to catalpol is

not too costly for the plant relative to its beneficial effect, the question arises, why constitutive levels of catalpol were not higher in the first place? One reason might be that aucubin is a more effective means of defense against pathogenic fungi (Marak et al., 2002a), which is in line with a recent finding by Marak et al. (2002b) that the proportion catalpol decreases when *P. lanceolata* is attacked by the fungus *Diaporthe adunca*. This suggests that *P. lanceolata*'s response to different attackers (pathogens or herbivores) is adaptive and that trade-offs affecting the effectiveness of defense against one or the other might exist. Autotoxicity or other functions of iridoid in plant metabolism may furthermore play a role. Experiments with both generalist and specialist herbivores and pathogens will be required to resolve the importance of the induced compared to the ontogenetic change in IGs and possible trade-offs in *P. lanceolata*'s defensive responses.

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ANTIHERBIVORE CHEMISTRY OF *Eucalyptus*—CUES AND DETERRENTS FOR MARSUPIAL FOLIVORES

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Abstract—Formylated phloroglucinol compounds (FPCs) are the single most important factor determining the amount of foliage that marsupial folivores eat from individual *Eucalyptus* trees. Folivores need to recognize which trees contain FPCs if they are to avoid them and forage efficiently, they are challenged by great diversity in the types and quantities of FPCs present, even within eucalypt species. We investigated the relationship between FPCs and terpenoids in species with both simple and complex FPC profiles and found strong positive correlations between terpenes generally, and several monoterpenes in particular, and FPCs. Terpene cues also indicated qualitative differences in trees' FPC profiles. We describe significant qualitative and quantitative variation in FPCs in several species that are important food sources for marsupial folivores. New discoveries include the fact that macrocarpals occur as two major, distinct groups and several new dimeric acylphloroglucinols from *Eucalyptus strzeleckii*. These patterns add to the chemical complexity of the foraging environment for folivores.

Key Words—Formylated phloroglucinol compounds, terpenoids, conditioned flavor aversion, Koala, *Phascolarctos cinereus*, jensenal, *Eucalyptus strzeleckii*, macrocarpals, sideroxylonals, 1,8-cineole.

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INTRODUCTION

Trees of the genus *Eucalyptus* L'Hérit. possess complex mixtures of plant secondary metabolites, including terpenoids, cyanogenic glycosides, hydrolyzable and condensed tannins, flavonoids, long chain ketones, and formylated phloroglucinol compounds (FPCs; Brophy and Southwell, 2002). Folivores of *Eucalyptus* need to recognize and limit their intake of these compounds, because their ability to tolerate or detoxify them sometimes falls short of their concentrations in foliage (Lawler et al., 1998b). This task becomes easier if sensory cues reliably indicate the presence and/or concentration of relevant compounds.

Among trees belonging to the *Eucalyptus* subgenus *Symphyomyrtus*, which accounts for more than half of all eucalypt species, the most important single variable determining feeding by marsupial folivores is the concentration of FPCs (Lawler et al., 2000; Wallis et al., 2002; B. D. Moore, unpublished data). In several species, such as *E. polyanthemos* Shau. (Lawler et al., 2000) and *E. microcorys* F. Muell. (Moore et al., in press), FPC concentrations are strongly correlated with concentrations of the monoterpene, 1,8-cineole. Because of this, Lawler et al. (1998a) suggested that marsupials develop conditioned flavor aversions to high concentrations of volatile terpenes because their flavor is consistently associated with the negative postingestive consequences of ingesting FPCs. Lawler et al. (1999b) showed that in experiments with isolated compounds, 1,8-cineole could be used by common brushtail possums as a cue to the concentrations of deterrent compounds. However, the situation in species examined to date may be misleadingly simple, because many eucalypts possess numerous monoterpenes and sesquiterpenes in addition to 1,8-cineole (Brophy and Southwell, 2002), as well as numerous FPCs in addition to, or in place of, sideroxylonal (Ghisalberti, 1996; Eschler et al., 2000; Eyles et al., 2003).

The simplest FPCs are fully substituted, formylated acylphloroglucinols, such as jensenone (Figure 1). These units form the basis of dimeric acylphloroglucinols, such as sideroxylonals, grandinal, and robustaol A, and form adducts, such as euglobals and macrocarpals, with mono- and sesquiterpenes. Although the terpene moiety suggests an obvious link between the concentrations of macrocarpals and euglobals and those of terpenoids, simple and dimeric FPCs do not share a biosynthetic precursor with terpenoids. Mono- and sesquiterpenes are predominately products of the deoxyxylulose phosphate, or mevalonate-independent pathway (Dewick, 2002), whereas the phenolic moiety of FPCs must arise from chalcone synthase-type enzymes as part of the shikimate pathway. One possible explanation for the covarying synthesis of FPCs and terpenes is that regulation of these processes is closely linked at the genetic level. The expression of terpenes in eucalypts is highly variable and has a strong genetic basis (Doran, 1992; Dungey et al., 2000; Dunlop et al., 2000), and variation in FPC concentrations in natural populations occurs at a scale too small to be explained by environmental

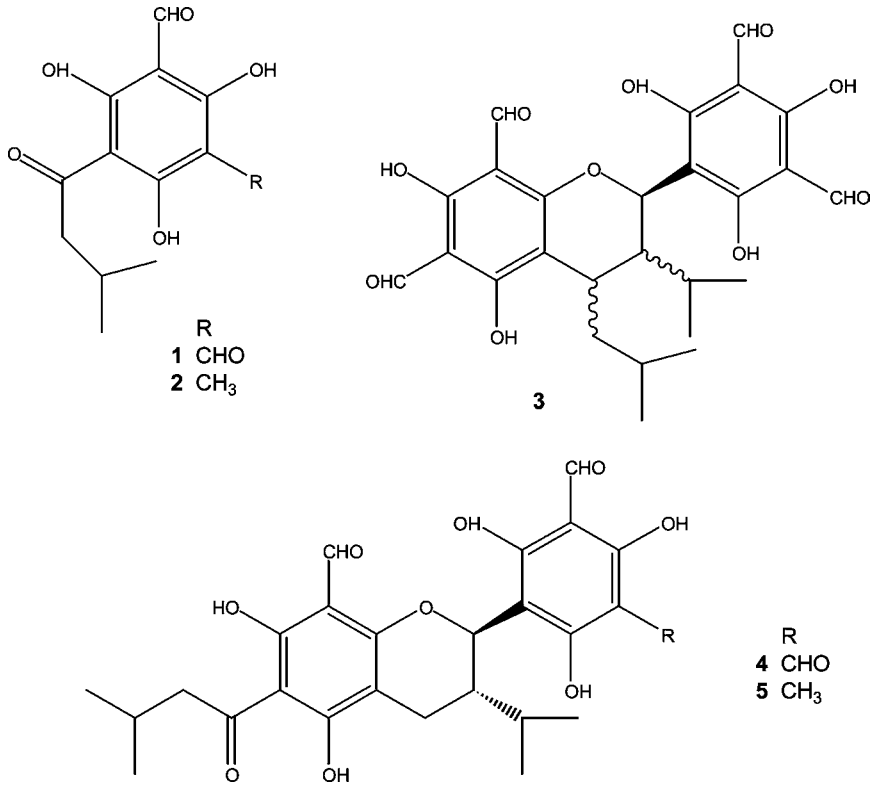

FIG. 1. Sideroxylonal (**3**), which occurs as 3 stereoisomers, is a dimer of jensenone (**1**), whereas grandinal (**4**) is a dimer of jensenone and grandinol (**2**). Structure **5** is a proposed grandinol dimer that would have a precise mass of 486.1890, consistent with ion peaks observed in *E. strzeleckii* extracts.

factors (R. Andrew, unpublished data). Given its interest from both ecological and biosynthetic perspectives, the link between FPCs and terpenes warrants further study. As a first step, this relationship must be better described, so we pursued this aim, using gas chromatography coupled to mass spectrometry (GC–MS) and high performance liquid chromatography (HPLC) to analyze and compare the terpene and FPC profiles from several *Eucalyptus* species. We considered two species that possess predominately sideroxylonal FPCs (*E. melliodora* A. Cunn. ex Schauer and *E. polyanthemos*) and two species possessing more complex FPC profiles (*E. globulus* Labill. and *E. viminalis* Labill.; Eschler et al., 2000).

Analytical limitations have restricted previous investigations of FPC content to eucalypt species that possess only sideroxylonal, so the qualitative variation in FPCs facing marsupial folivores remains largely unknown. Most FPCs have been

reported from only one or a few eucalypt species, and patterns of co-occurrence resulting from shared or overlapping biosynthetic pathways are poorly understood. Consequently, the other major aim of our study was to describe these patterns in detail for a large number of trees from several species. To achieve this, we used electrospray ionization, Fourier transform ion cyclotron mass spectrometry (ESI-FTMS), and HPLC to analyze foliage from *E. globulus*, *E. viminalis*, and *E. ovata* Labill. These species are widespread in south-eastern Australia and important food species for herbivorous marsupials. We also included a fourth species, *E. strzeleckii* K. Rule, for comparison with the closely related and sympatric *E. ovata*. *E. strzeleckii* is a recently described species (Rule, 1992) with a restricted distribution in the South Gippsland region of Victoria that, to our knowledge, has not been reported as a food species for vertebrate herbivores. All trees in this report were also used in an experiment, which will be reported elsewhere, to test the role of FPCs in determining the feeding preferences of koalas.

METHODS AND MATERIALS

To survey variation in FPCs, we collected foliage from 50 *E. globulus*, 29 *E. ovata*, 8 *E. strzeleckii*, and 51 *E. viminalis* trees in 1998 and 2002. Trees were selected randomly from Phillip Island, French Island, eastern Melbourne, and South Gippsland, in the state of Victoria. To study covariance in terpene and FPC concentrations, we collected foliage from a further 14 *E. globulus* and 15 *E. viminalis* from the same region, and 15 *E. melliodora* and 19 *E. polyanthemos* from near Canberra, Australian Capital Territory in 2002. We collected at least 100 g of mature foliage from the midcanopy of each tree and immediately froze samples at -20°C. Leaving aside 50 g subsamples from trees to be analyzed for terpenoids, we freeze-dried the samples and ground them to pass a 1-mm sieve in a Cyclotec 1093 cyclone mill (Tecator, Sweden). Further analyses were performed on solvent extracts made from this ground leaf material as follows.

We weighed 1.5 g of leaf material into a cellulose extraction thimble and refluxed this for at least 4 hr with 100 ml of 4:1 light petroleum spirit (40–60°C boiling point): acetone mixture in a Soxhlet extractor connected to a round-bottomed flask, heated to 85°C. We removed solvent from the flask by rotary evaporation at 50°C and transferred the crude extract into a glass vial using 4:1 dichloromethane:methanol. We determined the crude mass of extract after drying this solution under a stream of air for 24 hr and for a further 48 hr at room temperature.

Analysis of FPCs by ESI-FTMS. We randomly selected acetone–petrol extracts from 27 *E. globulus*, 26 *E. ovata*, 8 *E. strzeleckii*, and 30 *E. viminalis* samples collected in 1998 for analysis by ESI-FTMS. We dissolved $\sim$5 mg of dried crude extract in 1 ml MeOH and further diluted 10 μl in 1 ml MeOH. This solution

was continually infused at a flow rate of 1 μl min^{-1} into the external electrospray source (Analytica of Bradford, Bradford, CT) of a Bruker BioApex 47e Fourier transform ion cyclotron resonance mass spectrometer operating in negative ion mode with broadband [low resolution (6–10 k FWHM at m/z 500)] detection. Typically, the signal was averaged over 16 transients prior to Fourier transformation, requiring a data acquisition time of about 1 min, and the consumption of about 1 μg of the crude extract. The instrument was calibrated with sodium trifluoroacetic acid (TFA).

Analysis of FPCs by HPLC. We used HPLC to analyze all acetone–petrol extracts. We dissolved approximately 15 mg of extract in 5 ml of acetonitrile and analyzed between 12.5 and 25 μl of this solution with a Waters Alliance Model HPLC with photo diode array detector. The analytical column was a Wakosil 250 × 4 mm GL 3C18RS (SGE), and the column temperature was 37°C. Extracts were eluted under gradient conditions with 0.1% TFA in acetonitrile (A) and 0.1% TFA in water (B) as follows: 60% A/40% B for 5 min, linear gradient to 90% A/10% B at 60 min, hold for 10 min. Flow rate was 0.75 ml min^{-1}. We measured the peak response at 275 nm of 20 major peaks from the resulting chromatographs (Table 1).

TABLE 1. MAJOR HPLC CHROMATOGRAPH PEAKS FROM EUCALYPT FOLIAGE EXTRACTS

Retention time (min)	Formula	Identity	Standard curve?
11			
13	$C_{28}H_{42}O_7$	**Macrocarpal I**	Y
17	$C_{28}H_{42}O_7$	**Macrocarpal J**	
25	$C_{19}H_{18}O_5$	**Eucalyptin**	Y
27	$C_{28}H_{38}O_7$	Macrocarpal N?	
28	$C_{28}H_{38}O_7$	**Eucalyptone**	Y
32	$C_{28}H_{40}O_6$	**Macrocarpal A**	Y
34	$C_{28}H_{40}O_6$	Macrocarpal	Y
35	$C_{28}H_{40}O_6$	Macrocarpal	
37	$C_{28}H_{40}O_6$	**Macrocarpal B**	Y
39	$C_{28}H_{40}O_6$	Macrocarpal	
42	$C_{28}H_{40}O_6$	Macrocarpal	
43	$C_{26}H_{28}O_{10}$	**Grandianal**	Y
44	$C_{26}H_{28}O_{10}$	**Sideroxylonal A**	Y
45	$C_{26}H_{28}O_{10}$	**Sideroxylonal C**	Y
47	$C_{28}H_{40}O_6$	Macrocarpal	
53	$C_{28}H_{40}O_6$	**Grandinal**	Y
60	$C_{26}H_{28}O_{10}$	Jensenal	Y
66	$C_{25}H_{30}O_9$	Monoterpene euglobal CHO	
70	$C_{23}H_{30}O_5$	**Macrocarpal G**	Y

We identified macrocarpals G, A, and B, the macrocarpal eucalyptone, sideroxylonals A and C, and grandinal on the basis of their coelution with authentic standards and by comparison of HPLC-UV data. We also collected fractions corresponding to most major peaks, which we analyzed with ESI-FTMS, as described above, to determine precise masses of the compounds. We collected between 2 and 6 mg each of compounds eluting at 13, 17, 25, 34, and 60 min from crude extract injected onto a Waters 300 × 7.8 mm Preparative NovaPak HRC18 column operated under similar gradient conditions to those described above, at a flow rate of 3.0 ml min^{-1}. ^{1}H NMR spectra of the 13, 17, and 25 min peaks confirmed identities as macrocarpals I and J and the flavonoid eucalyptin (Horn and Lamberton, 1963; Osawa et al., 1996). We determined extinction coefficients for a number of these compounds (Table 1). Several extracts containing compounds of interest were analyzed by MS and MS–MS, providing additional identification of these compounds as FPCs. We did not determine extinction coefficients for the compounds eluting as smaller peaks at 17, 27, 35, 39, 42, and 47 min, but used averaged extinction coefficients from other compounds with the same molecular formula to quantify them.

Extraction and Analysis of Terpenes. We used steam distillation to extract foliar terpenes from ∼50 g samples of frozen leaves. We estimated the dry matter content of this sample by drying a second portion of the leaves at 80°C for 48 hr. Our procedure was true steam distillation and not hydrodistillation, which is most commonly used to study *Eucalyptus* terpenes. Dunlop et al. (2000) showed that hydrodistillation alters the composition of distilled oils relative to those isolated by either vacuum distillation or steam distillation, probably because of rearrangements induced by pH changes in the water in which the leaves are distilled. We modified previously described methods (Foley et al., 1987) by suspending the leaves above boiling water on stainless steel mesh inside a 2 l flanged flask. Water returning from the collection burette was channelled via a funnel back to the base of the flask so that it did not drip though the leaf mass. The volume of oil recovered was measured, and the oil was dried over sodium sulphate and stored in vials with nitrogen at −20°C until it was analyzed.

Analytical gas chromatography (GC) (Shimadzu GC17A with FID) was used to quantify terpenes. Samples of distilled oil were injected on a column of DB-Wax (60 m × 0.5 mm × 1 μm) programmed to ramp from 50 to 220°C at 3°C min^{-1} with helium as a carrier gas. FID integrations were performed on a SMAD electronic integrator. GC–MS was used to identify oil components and performed on a VG Quattro mass spectrometer operating at 70 eV ionization energy. The GC column used was a DB-Wax (60 m × 0.32 mm × 0.25 μm) ramped from 35 to 220°C at 3°C min^{-1} with helium as carrier gas. Mono- and sesquiterpenes were identified by their identical GC retention times relative to known compounds and by comparison of their mass spectra with either known compounds or published

spectra (Stenhagen et al., 1974; Heller and Milne, 1978, 1980, 1983; Swigar and Silverstein, 1981; Adams, 1995; Joulain and König, 1998).

Statistical Analyses. To identify patterns of covariance among FPC constituents measured by HPLC, we constructed a correlation matrix describing pairwise correlations among the areas of all 20 peaks measured from chromatographs. Significance levels of correlation coefficients were adjusted by the serial Bonferroni procedure. For each species analyzed by GC–MS, we considered all possible pairwise correlations between the concentrations of independently occurring FPCs or groups of FPCs and all measured terpenes to identify the strongest correlations. Where we wished to further investigate the relationships between individual terpene and FPC groupings, we performed model II-type simple linear regressions, using major axis regression calculated by the computer program "Model II Regression" (Legendre, 2001).

RESULTS

Detection of Qualitative and Quantitative Variation in FPCs by ESI-FTMS. Most prominent ions detected from petrol–acetone extracts by ESI-FTMS were consistent with the molecular formulae of previously reported FPCs, free fatty acids or triterpene acids (Table 2). Several smaller peaks could be attributed to flavones and arenic and β-triketones. We detected masses consistent with most published FPCs, as well as several of the triterpene and sesterterpene FPCs and disubstituted monoterpene and sesquiterpene FPCs proposed by Eyles et al. (2003). The strongest ion peak from *E. strzeleckii* was at m/z 473.1798. Tandem MS analysis of this compound gave a strong product ion at m/z 237 and a weaker product ion at m/z 223, consistent with the structure of **6**, fragmenting at points indicated by (a) and (b) in (Figure 2). This compound, jensenal, has previously been isolated and characterized from *E. jensenii* Maiden (Midori Takasaki, personal communication). ^{1}H NMR and ^{13}C NMR spectra of the compound from *E. strzeleckii* were consistent with those from jensenal. *E. strzeleckii* extracts also produced strong peaks at m/z 488.1682 and 490.1859, which gave MS–MS spectra dominated by an m/z 237 product ion. The first mass is consistent with a compound recently reported from *E. saligna* (**8**, Figure 2; Mitaine-Offer et al., 2003), and the second with **7**. The smaller peak detected from *E. strzeleckii* at m/z 485.1811 may be **5** (Figure 1), a grandinol dimer.

ESI-FTMS revealed three basic FPC profiles (Figure 7). First, the spectra of all *E. globulus* and *E. viminalis* and of many *E. ovata* trees were dominated by macrocarpals, with other FPC peaks (including sideroxylonal) much less intense in comparison. Second, a number of *E. ovata* trees produced much stronger sideroxylonal peaks, although large macrocarpal peaks were still present. Third, all

TABLE 2. MAJOR ION PEAKS DETECTED BY ESI-FTMS FROM EUCALYPT FOLIAGE EXTRACTS

Calculated (m/z)	Measured (m/z)	Compound	Occurrence[a]				Mean r.i. (%)[b]	Reference[c]
			Eg	Es	Eo	Ev		
Formylated phloroglucinol compounds								
453.26	453.26	Macrocarpal G, sesquiterpene Euglobals	+++	+++	+++	+++	57.4	1,2
485.25	485.25	Eucalyptone, Macrocarpal N	+++	+++	+++	+++	20.7	3,4
471.27	471.27	Macrocarpals A,B,D,E,F,H,K,L,M,O	+++	+++	++	+++	68.2	1,5,6,3
385.20	385.20	Monoterpene euglobals	+++	+++	++	+++	8.2	2
675.46	675.46	Triterpene alcohol FPCs	++	−	++	+++	3.8	7
401.20	401.20	Oxidized monoterpene euglobals	++	+++	++	++	1.3	8
499.16	499.16	Sideroxylonal, grandinal	++	+++	++	+	11.2	9,10,11
657.45	657.45	Triterpene HCO FPCs	++	−	+	++	2.4	7
473.18	473.18	Jensenal (**6**)	+	+++	+	++	15.6	
589.39	589.38	Sesterterpene HCO FPCs	++	−	+	++	0.8	7
489.29	489.28	Macrocarpal I, J	++	+++	++	+	8.0	5
607.40	607.40	Sesterterpene alcohol FPCs	++	−	+	++	0.9	7
251.09	251.09	Grandional	+	+++	++	++	1.2	12
403.21	403.21	Monoterpene alcohol/ether FPCs	+	+++	++	++	1.5	7
703.35	703.35	Disubst, sesquiterpene HCO FPCs	+	++	++	+	4.4	7
485.18	485.18	Possible structure **5**	+	++	+	+	1.4	
635.29	635.28	Disubst. monoterpene HCO FPCs	−	+++	+	+	2.7	7
489.18	489.18	Possible strucutre **7**	+	+++	+	+	12.1	
487.16	487.16	Structure **8**	−	+++	+	−	7.2	
721.36	721.36	Disubst. sesquiterpene alcohol FPCs	−	+++	+	−	2.7	7

TABLE 2. CONTINUED

Calculated (m/z)	Measured (m/z)	Compound	Occurrence[a]				Mean r.i. (%)[b]	Reference[c]
			Eg	Es	Eo	Ev		
Flavones								
311.09	311.09	Sideroxylin	++	++	+++	+++	2.7	13,14
297.08	297.08	8-demethylsideroxylin	++	+	++	++	1.1	14
Ketones								
265.14	265.15	Apodophyllone, leptosermone	++	+++	++	+++	2.0	15
279.16	279.16	Torquatone, isotorquatone	+	+	+	+	0.7	15,16
251.13	251.13	Flavesone	+	−	−	+	1.0	17
235.10	235.10	Agglomerone	+	+	+	+	0.4	17

[a] +++ = ion present in 100% of trees; ++ = 50–100%; + = 0–50%; − = 0%; symbols are in bold type if mean r.i. > 10% for that species; Eg = *E. globulus*, Es = *E. strzeleckii*, Eo = *E. ovata*, Ev = *E. viminalis*.

[b] Mean relative intensity (r.i.) calculated from all non-zero values. r.i. does not accurately indicate relative concentrations of these compounds.

[c] Previous reports from *Eucalyptus*: 1 = Nishizawa et al., 1992; 2 = Amano et al., 1981; 3 = Shibuya et al., 2001; 4 = Osawa et al., 1995; 5 = Yamakoshi et al., 1992; 6 = Terada et al., 1999; 7 = Eyles et al., 2003; 8 = Eschler et al., 2000; 9 = Satoh et al., 1992; 10 = Eschler and Foley, 1999; 11 = Singh et al., 1997; 12 = Yoshida et al., 1988; 13 = Mitaine-Offer et al., 2003; 14 = Sarker et al., 2001; 15 = Menut et al., 1999; 16 = Brophy et al., 1996; 17 = Brophy and Southwell, 2002.

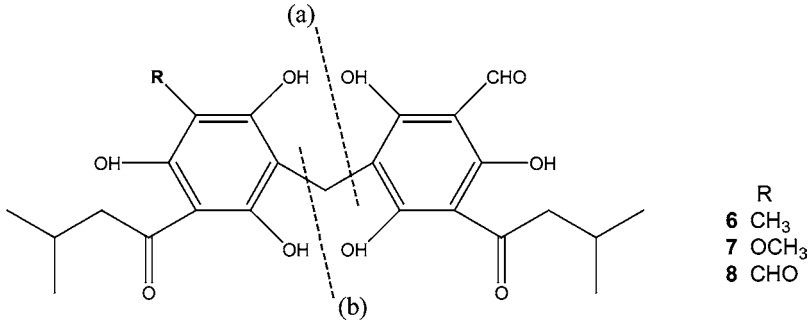

FIG. 2. Jensenal (**6**) and suggested structures for the compounds of precise mass 490.1859 (**7**) and 488.1682 (**8**) observed in *E. strzeleckii* extracts. The fragment to the right of the dashed line (a) has MW 237, which was observed in tandem MS analysis of these three compounds. The fragment of **6** to the left of (b) has MW 223, which was observed in tandem MS analysis of that compound.

E. strzeleckii trees produced small macrocarpal peaks, but large peaks attributable to jensenal and sideroxylonal and/or grandinal, as well as the peaks discussed above, which were absent from the other species.

Detection of Qualitative and Quantitative Variation in FPCS by HPLC. Example HPLC chromatographs are shown in Figure 3. Twelve of the 20 peaks quantified were attributable to macrocarpals, 2 to sideroxylonals, 2 to tautomers of grandinal, and 1 to jensenal. Only one of these major peaks was a euglobal, one was eucalyptin, and one was not identified.

Patterns of covariance among the 20 major peaks are shown in (Figure 4). Strong correlations indicate that certain peaks always co-occurred in constant proportions with other peaks. We refer to the group comprising the 70, 32, 37, 35, 39, 47, 28, and 27 min peaks (listed in order of decreasing peak size) as "group 1" macrocarpals, and the group consisting of the 34, 13, 17, and 42 min peaks as "group 2" macrocarpals. The isomers sideroxylonal A and C also occurred in a fixed ratio, and grandinal is a tautomer (Singh et al., 1997) that always produces two equal peaks. Although Figure 4 shows that grandinal and jensenal peak sizes are strongly correlated, the ratio describing their relative proportions is more variable than those for other groupings, and the significant correlation coefficient partly reflects these compounds' co-occurrence in all *E. strzeleckii* trees. The relative concentrations of compounds in each group are illustrated in Figure 5.

Total FPC concentration and the concentration of individual FPCs and groups of FPCs were highly variable in all species except *E. strzeleckii*, although this exception may reflect the smaller number of *E. strzeleckii* trees analyzed (Figure 6). HPLC confirmed and distinguished more clearly the existence of three basic FPC profiles, showing that macrocarpals were generally absent from the

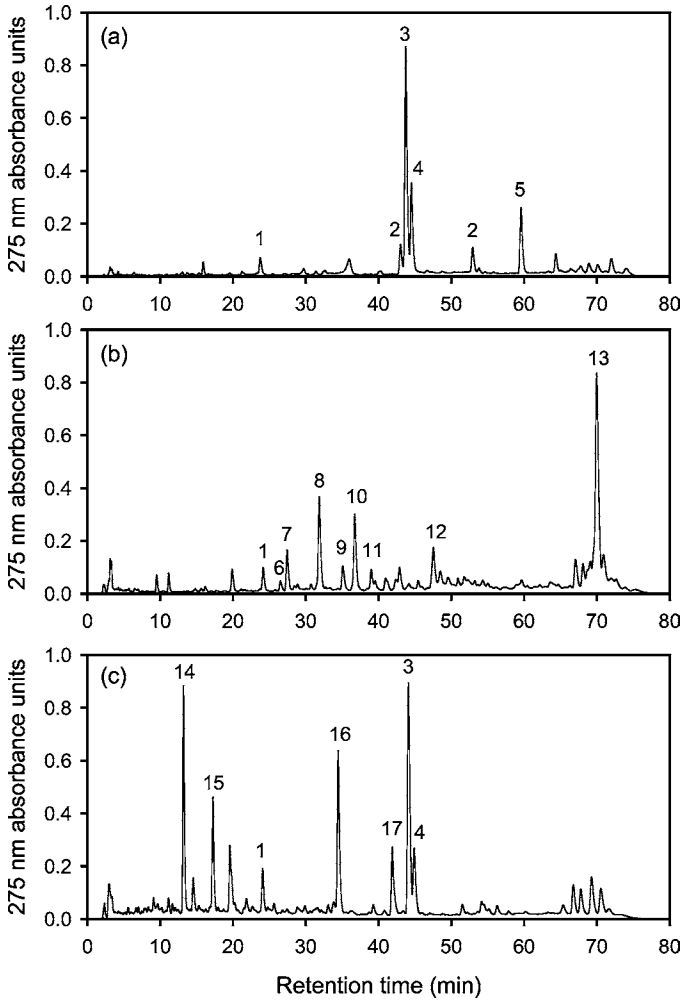

FIG. 3. HPLC chromatographs from foliage extracts of (a) *E. strzeleckii* (b) *E. viminalis* with group 1 macrocarpals and (c) *E. globulus* with group 2 macrocarpals and sideroxylonal. 1: eucalyptin, 2: grandinal, 3: sideroxylonal A, 4: sideroxylonal C, 5: jensenal, 6: macrocarpal ($R_t = 27$ min); 7: eucalyptone; 8: macrocarpal A; 9: macrocarpal ($R_t = 35$ min); 10: macrocarpal B; 11: macrocarpal ($R_t = 39$ min); 12: macrocarpal ($R_t = 47$ min); 13: macrocarpal G; 14: macrocarpal I; 15: macrocarpal J; 16: macrocarpal ($R_t = 34$ min); 17: macrocarpal J ($R_t = 42$ min).

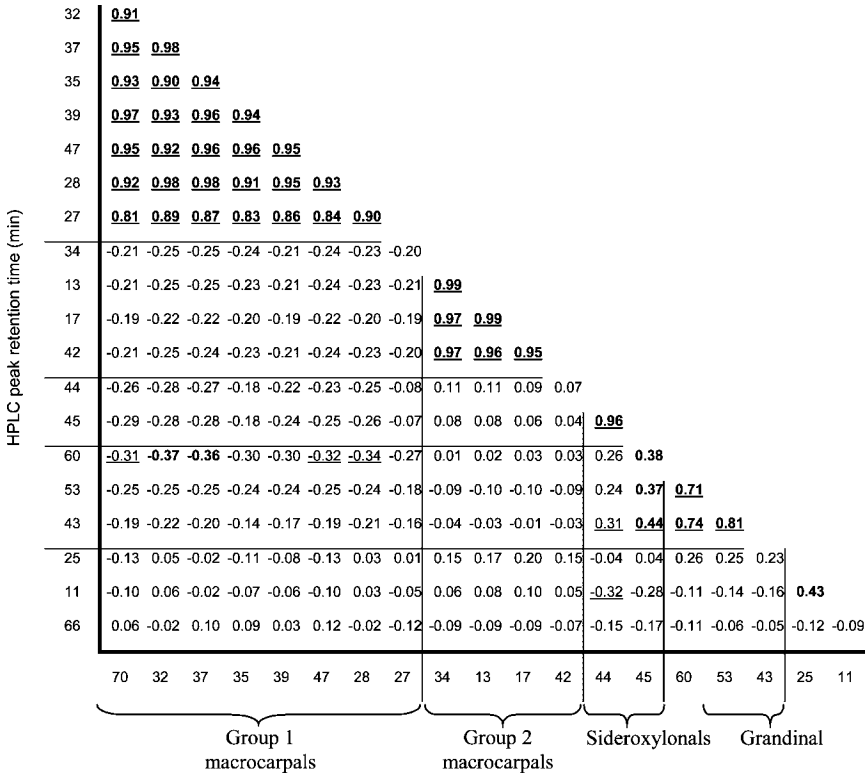

FIG. 4. Correlation matrix of HPLC peak responses from 130 eucalypt foliage extracts. (Correlation coefficients in bold and underlined are significant at $P < 0.001$; in bold at $P < 0.01$ and underlined, normal weight at $P < 0.05$ after sequential Bonferroni adjustment.)

sideroxylonal-rich *E. ovata* chemotype and *E. strzeleckii*. Macrocarpal-rich trees were generally dominated by "group 1" macrocarpals, however, in some trees, they were replaced by "group 2" macrocarpals or by a mixture of the two groups (Figure 6).

Comparison of HPLC and ESI-FTMS Results. Although ESI-FTMS detected macrocarpals in all trees, they were not detected by HPLC in sideroxylonal-dominated *E. ovata* or *E. strzeleckii*. Conversely, the intensity of ESI-FTMS sideroxylonal peaks was much less than its true concentration would suggest (Figure 7). We sought to quantify the difference between macrocarpal and sideroxylonal responses on the ESI-FTMS system by coinjecting authentic standards of sideroxylonal A and macrocarpal G in four different proportions. Relative to the quantity of compound injected, the macrocarpal G peaks were more than an

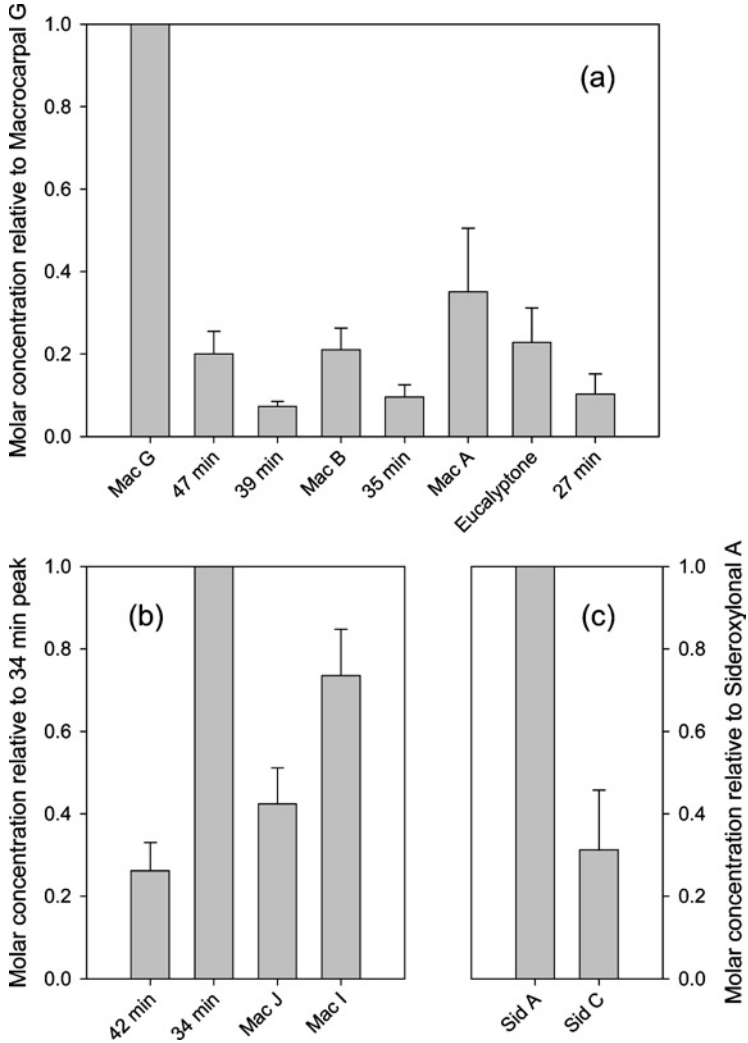

FIG. 5. Mean relative molar concentrations of (a) "group 1" macrocarpals ($N \geq 97$); (b) "group 2" macrocarpals ($N \geq 14$); (c) sideroxylonals ($N = 115$) measured by HPLC. Error bars represent 1 standard deviation.

order of magnitude greater than sideroxylonal A peaks. In the trees we analyzed, ESI-FTMS indicated the concentrations of differently-sized macrocarpals (e.g., macrocarpals with the formula $C_{28}H_{42}O_7$ and $C_{28}H_{38}O_5$; Figure 7) relative to one another, but not the relative concentrations of more structurally dissimilar groups, such as macrocarpals and sideroxylonals.

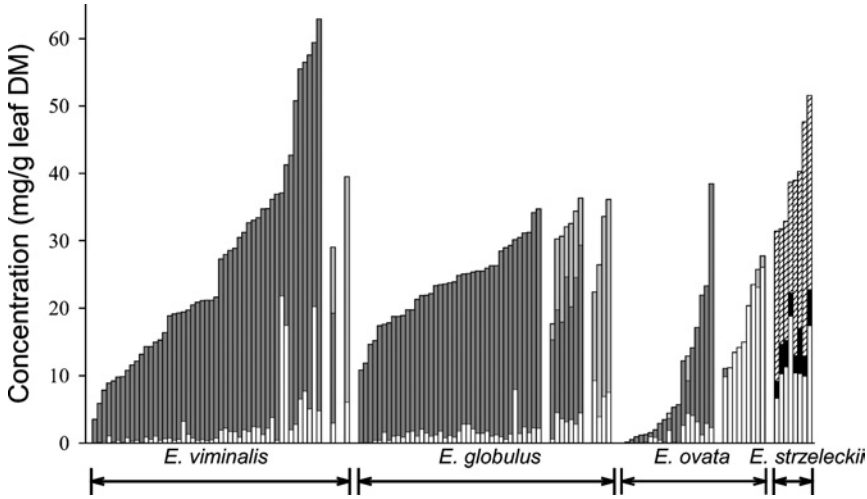

FIG. 6. Foliar concentrations of sideroxylonals (white bars), "group 1" macrocarpals (dark gray), "group 2" macrocarpals (light grey), grandinal (black), and jensenal (cross-hatched) in four eucalypt species, measured by HPLC. Bars representing different species and qualitatively dissimilar trees have been separated by gaps.

Terpene and FPC Relationships. We identified 76 terpenes in the four species analyzed (Table 3). All species were dominated by monoterpenes, particularly 1,8-cineole, with smaller amounts of α-pinene, limonene, p-cymene, and the sesquiterpene, globulol. Within each species, the oils obtained were mostly qualitatively similar, however, two *E. melliodora* trees had unusual terpene profiles—they produced moderate oil yields and were dominated by p-cymene and spathulenol, but contained little 1,8-cineole. One unusual *E. polyanthemos* tree possessed concentrations of most terpenes that were typical for that species, but also possessed a very high concentration of β-phellandrene. The FPC profiles of both *E. melliodora* and *E. polyanthemos* were dominated by sideroxylonals, but *E. melliodora* also contained small amounts of grandinal and some *E. polyanthemos* trees possessed small concentrations of "group 1" macrocarpals.

Sideroxylonal and total terpene concentrations were positively correlated in *E. melliodora* ($r^2 = 0.34$, $P = 0.02$), however, the two trees with unusual terpene profiles deviated from the general trend (Figure 8). We found stronger correlations between sideroxylonal and 1,8-cineole ($r^2 = 0.67$, $P < 0.001$) and sideroxylonal and limonene ($r^2 = 0.77$, $P < 0.001$; Figure 8). The correlation between sideroxylonal and total terpene concentration was stronger in *E. polyanthemos* ($r^2 = 0.62$, $P < 0.001$), although a tree with an unusual terpene profile formed an outlier in this species too (Figure 8). Sideroxylonal concentration in *E. polyanthemos* was

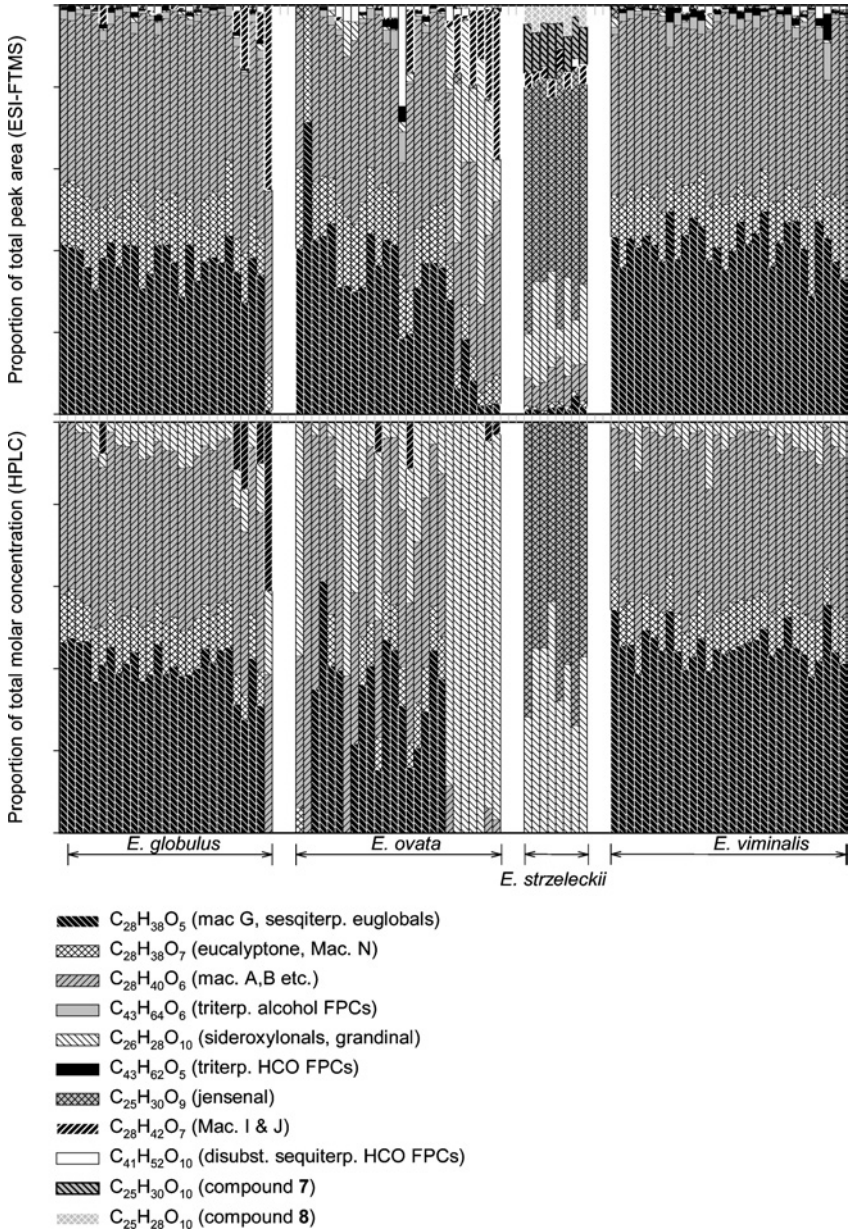

FIG. 7. Relative proportions of ESI-FTMS peak intensities (above) and relative molar concentrations determined by HPLC (below) of major FPC compounds in four eucalypt species.

TABLE 3. TERPENOIDS[a] IDENTIFIED BY GC-MS FROM STEAM-DISTILLED FOLIAGE EXTRACTS FROM *E. melliodora* (EM), *E. polyanthemos* (EP), *E. globulus* (EG), AND *E. viminalis* (EV)

	Em		Ep		Eg		Ev	
1,8-Cineole	**91.6**	(2.4–199.4)	**85.3**	(0–323.3)	**130.2**	(51.3–266.8)	**82.4**	(1.2–245.1)
α-Pinene	**15.0**	(1.6–29.5)	**5.9**	(0–24.3)	**40.7**	(16.8–79.1)	**33.8**	(0.1–137.5)
Limonene	**7.3**	(0.7–14.8)	**6.3**	(0–15.5)	**9.1**	(2.1–21.6)	**8.2**	(0.3–24.3)
p-Cymene	**6.1**	(0.5–31.9)	**4.6**	(0.4–18.2)	**4.5**	(0.3–14.8)	**9.3**	(0.6–27.1)
globulol	**1.7**	(0.1–8.7)	**1.2**	(0–3.3)	**8.0**	(0–15.2)	**12.4**	(6.0–21.3)
α-Terpineol	**3.4**	(0.4–7.4)	**5.4**	(0–23.9)	**2.4**	(0.5–7.7)	**2.3**	(0.1–16.2)
β-Eudesmol	—		—		**6.0**	(0–30.8)	—	
trans-Pinocarveol	**2.2**	(0–7.0)	**0.5**	(0–1.7)	**5.0**	(0.7–12.8)	**2.6**	(0–12.6)
Aromadendrene	**0.3**	(0.1–1.0)	**0.5**	(0–1.3)	**4.7**	(0.2–11.0)	**2.6**	(0.3–11.1)
γ-Terpinene	**0.8**	(0.0–6.9)	**1.1**	(0–6.0)	**0.6**	(0–3.8)	**4.9**	(0.1–24.6)
β-Phellandrene	**0.3**	(0–2.1)	**5.6**	(0–78.4)	—	(0–0.2)	**0.1**	(0–0.7)
Terpinyl acetate	**2.4**	(0–7.2)	**3.5**	(0–11.3)	—		—	
Spathulenol	**3.5**	(0.1–23.2)	**1.8**	(0.0–9.9)	**0.5**	(0–1.2)	**0.5**	(0.1–1.0)
α-Phellandrene	**1.7**	(0–11.9)	**2.5**	(0–18.7)	**0.4**	(0–1.5)	**0.8**	(0–6.5)
Terpinen-4-ol	—		—	(0–1.1)	**0.2**	(0–1.5)	**5.4**	(0–10.8)
Pinocarvone	**0.5**	(0.1–1.4)	**0.1**	(0–0.5)	**4.3**	(0.6–10.0)	**0.8**	(0–5.5)
Epiglobulol	—		—	(0–0.2)	**1.6**	(0–3.2)	**2.5**	(0–5.2)
β-Caryophyllene	**1.9**	(0.1–10.5)	**1.1**	(0–10.8)	**0.2**	(0–0.9)	**0.2**	(0–0.5)
Viridiflorene	**0.6**	(0–2.9)	**0.3**	(0–1.5)	**1.1**	(0–2.3)	**1.6**	(0.6–2.6)
$C_{15}H_{26}O_3$	—		—		**0.7**	(0–1.6)	**1.0**	(0–1.8)
α-Eudesmol	—		—		**1.8**	(0–13.3)	—	
Viridiflorol	**1.6**	(0–10.8)	**0.5**	(0–2.8)	**0.6**	(0–2.8)	**0.4**	(0–1.2)
Bicyclogermacrene	**1.8**	(0.1–15.0)	**1.1**	(0–6.7)		—	—	
γ-Eudesmol	—		—		**1.4**	(0–10.3)	—	
allo-Aromadendrene	**0.4**	(0–2.1)	**0.3**	(0–1.0)	**1.0**	(0.2–1.6)	**1.0**	(0–2.0)
trans-Menth-1(7),8-diene-2-ol	**0.5**	(0.1–1.4)	**0.4**	(0–1.4)	**1.1**	(0.2–2.4)	**0.4**	(0–1.4)

$C_{15}H_{26}O_2$	—		—		**0.6**	(0–1.5)	**0.5**	(0–0.8)
α-Bulnesene	**0.5**	(0.1–1.4)	**1.0**	(0–6.5)	**0.6**	(0.2–1.3)	—	
β-Pinene	**0.1**	(0–0.4)	**0.2**	(0–0.7)	**1.1**	(0.2–2.2)	**0.7**	(0–2.6)
Cubeban-11-ol	**0.8**	(0–5.9)	**0.3**	(0–1.0)	**0.4**	(0–0.7)	**0.5**	(0.2–1.0)
Isoamyl isovalerate	**0.6**	(0–1.8)	**0.4**	(0–3.2)	**0.1**	(0–0.6)	**0.5**	(0–1.8)
$C_{15}H_{26}O$	**0.6**	(0–4.5)	**0.2**	(0–1.7)	—		—	
cis-Menth-1(7),8-diene-2-ol	**0.4**	(0–1.2)	**0.2**	(0–0.8)	**0.7**	(0–1.5)	**0.3**	(0–1.0)
$C_{15}H_{26}O$	**0.5**	(0–4.6)	**0.3**	(0–2.8)	—		—	
Thymol	—		—		—		**0.6**	(0–2.8)
$C_{15}H_{26}O$	**0.4**	(0–3.3)	**0.1**	(0–0.5)	—		—	
Borneol	**0.4**	(0.1–1.1)	**0.3**	(0–3.2)	**0.2**	(0–0.6)	—	
Ledol	**0.2**	(0–1.1)	**0.1**	(0–0.8)	**0.2**	(0–0.4)	**0.4**	(0.1–0.6)
Neral	—		—		—		**0.4**	(0.1–0.9)
trans-Menth-1,8-dien-6-ol	**0.1**	(0–0.3)	**0.2**	(0–2.0)	**0.4**	(0–1.3)	**0.1**	(0–0.5)
Myrcene	—	(0–0.2)	**0.3**	(0–4.6)	**0.2**	(0–1.7)	**0.3**	(0–1.7)
Terpinolene	**0.1**	(0–0.4)	**0.2**	(0–1.0)	**0.2**	(0–0.6)	**0.2**	(0–1.3)
α-Gurjunene	**0.1**	(0–0.6)	—		**0.2**	(0–1.2)	**0.3**	(0–0.9)
Myrtenol	—		—		**0.1**	(0–0.3)	**0.2**	(0–0.5)
Ketone 142	—		—		**0.4**	(0–1.9)	—	
Palustrol	**0.2**	(0–1.3)	**0.2**	(0–1.6)	**0.1**	(0–0.2)	**0.1**	(0–0.2)
Carvone	—		—		**0.3**	(0–0.7)	—	
Caryophyllene oxide	—		**0.2**	(0–4.6)	—		—	
p-Cymene-8-ol	—	(0–0.2)	—	(0–0.4)	**0.2**	(0–0.8)	**0.1**	(0–0.5)
β-Gurjunene	—		—		**0.3**	(0–1.8)	—	
Z-β-Ocimene	—		—		—		**0.2**	(0–0.7)
Menth-1-en-7-al	—	(0–0)	**0.2**	(0–3.8)	—		—	
δ-Terpineol	**0.1**	(0–0.6)	**0.1**	(0–0.8)	**-**		**0.2**	(0–0.4)
Carvacrol	—		—		—		**0.2**	(0–0.4)
$C_{10}H_{14}$	—		—		**0.2**	(0–0.5)	—	
Fenchol	**0.2**	(0–0.5)	**0.1**	(0–1.1)		—	—	
E-β-Ocimene	**0.1**	(0–0.2)	**0.1**	(0–0.4)	—		**0.1**	(0–0.3)

TABLE 3.CONTINUED

	Em		Ep		Eg		Ev	
Piperitol	—	—			**0.1**	(0–0.4)	—	
cis-Menth-1,8-dien-6-ol	—	(0–0.2)	—	(0–0.2)	**tr**		—	(0–0.1)
Cryptone	—		**0.1**	(0–2.9)	—		—	
α-Terpinene	—	(0–0.1)	**0.1**	(0–1.4)	**tr**		**0.1**	(0–0.3)
α-Campholenic aldehyde	—		—		**0.1**	(0–0.9)	—	
Isovaleraldehyde	—		—	(0–1.3)	**0.1**	(0–0.6)	**0.1**	(0–1.0)
Humulene	**0.1**	(0–0.5)	**0.1**	(0–0.5)	—		—	
Camphene	**0.1**	(0–0.2)	**tr**		**0.1**	(0–0.2)	—	(0–0.3)
Phenylethyl isovalerate	—		—		**0.1**	(0–0.4)	—	
α,*p*-Dimethyl styrene	—		—		**tr**		**0.1**	(0–0.4)
α-Fenchene		(0–0.1)	**tr**	**tr**			—	
$C_{10}H_{14}$	—		—		**0.1**	(0–0.2)	—	
hex-3-en-1-ol	—		—		**0.1**	(0–0.2)	—	
Sabinene	—		**0.1**	(0–2.2)	—		—	
Ethyl-isovalerate	—		—		—		**tr**	
Isoamyl acetate	—			(0–0.2)	**tr**		—	
Isobutyl Isobutyrate	—		—		—		**0.1**	(0–0.1)
α-Thujene	—			(0–0.6)	—		—	
Cuminal	—			(0–0.3)	—		—	

[a] All values are concentrations (mg/g DM), mean concentrations are in bold type, and minima and maxima are provided in brackets.

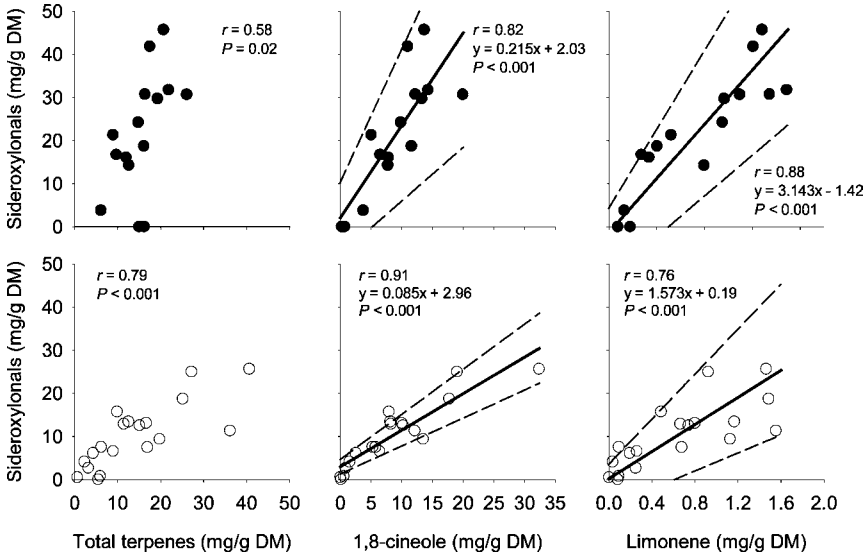

FIG. 8. Plotted FPC and terpene concentrations in 15 *E. melliodora* trees (closed circles) and in 19 *E. polyanthemos* trees (open circles). Listed on each figure are Pearson's correlation coefficient (r) and the associated significance level (P). Solid lines indicate major axis regressions and dashed lines 95% confidence intervals.

most strongly correlated with 1,8-cineole ($r^2 = 0.83$, $P < 0.001$) and limonene ($r^2 = 0.58$, $P < 0.001$; Figure 8). The major axis regression coefficient (x = cineole, y = sideroxylonal) was significantly greater for *E. melliodora* (95% confidence interval: 0.125–0.308) than for *E. polyanthemos* (0.065–0.105), reflecting the higher ratio of sideroxylonal: cineole in the former species.

In *E. globulus* (Figure 9), total FPC concentration was positively correlated with total terpenes ($r^2 = 0.67$, $P < 0.001$), 1,8-cineole ($r^2 = 0.72$, $P < 0.001$), and limonene ($r^2 = 0.66$, $P < 0.001$). In this species, the positive correlation between total terpenes and total macrocarpals was weaker ($r^2 = 0.53$, $P = 0.03$), but that between total terpenes and sideroxylonals was stronger than for FPCs overall ($r^2 = 0.79$, $P < 0.001$). In *E. viminalis* (Figure 10), total terpene concentration was a better predictor of total FPCs ($r^2 = 0.79$, $P < 0.001$) than 1,8-cineole ($r^2 = 0.59$, $P < 0.001$). Total terpene concentration was also correlated with "group 1" macrocarpals ($r^2 = 0.67$, $P < 0.001$) and sideroxylonals ($r^2 = 0.45$, $P = 0.006$), but not with "group 2" macrocarpals, which occurred in less than half of the trees ($r^2 = 0.13$, $P = 0.19$). The major axis regression coefficient (x = total terpenes, y = total FPCs) for *E. globulus* was significantly less (95% confidence interval: 0.244–0.643) than that for *E. viminalis* (0.811–1.524). The y-intercept of the *E. globulus* regression was greater than zero.

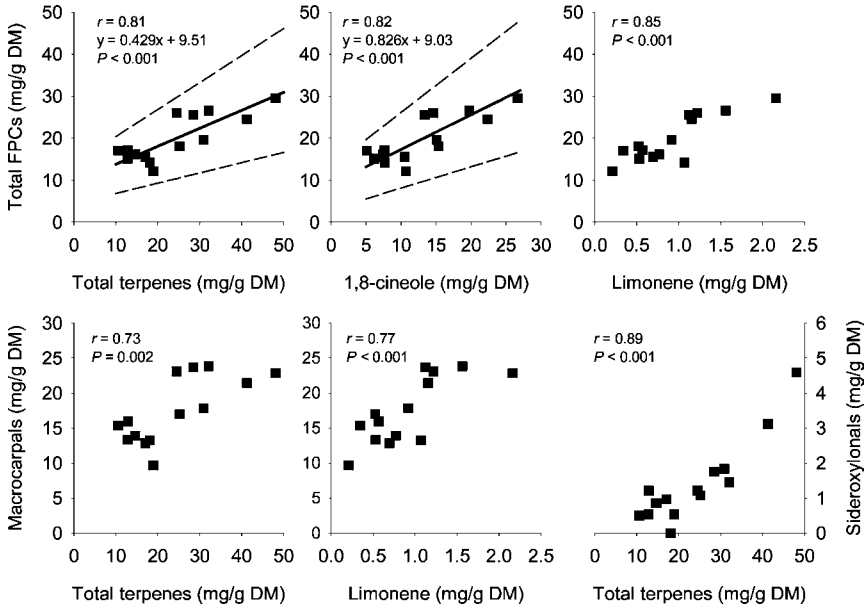

FIG. 9. Plotted FPC and terpene concentrations in 14 *E. globulus* trees. Listed on each figure are Pearson's correlation coefficient (r) and the associated significance level (P). Solid lines indicate major axis regressions and dashed lines 95% confidence intervals.

Of the 14 *E. globulus* trees, two possessed "group 2" macrocarpals but no "group 1" macrocarpals. For eight, the reverse was true, and the other four contained both groups. Among the small number of trees considered, higher concentrations of total terpenes and of 1,8-cineole corresponded to higher "group 2" macrocarpal concentrations when they were present (Figure 11). "Group 2" macrocarpal concentration was also strongly correlated with the covarying concentrations of β-, α-, and γ-eudesmol. We only detected eudesmols in *E. globulus* possessing "group 2" macrocarpals. Few strong correlations occurred between "group 1" macrocarpals and individual terpene concentrations in *E. globulus*, and the strongest of these, between "group 1" macrocarpals and globulol, only occurred amongst trees possessing "group 2" macrocarpals (Figure 11).

DISCUSSION

The great inter- and intraspecific variation in the types and concentrations of FPCs in the species that we considered has important implications for marsupial

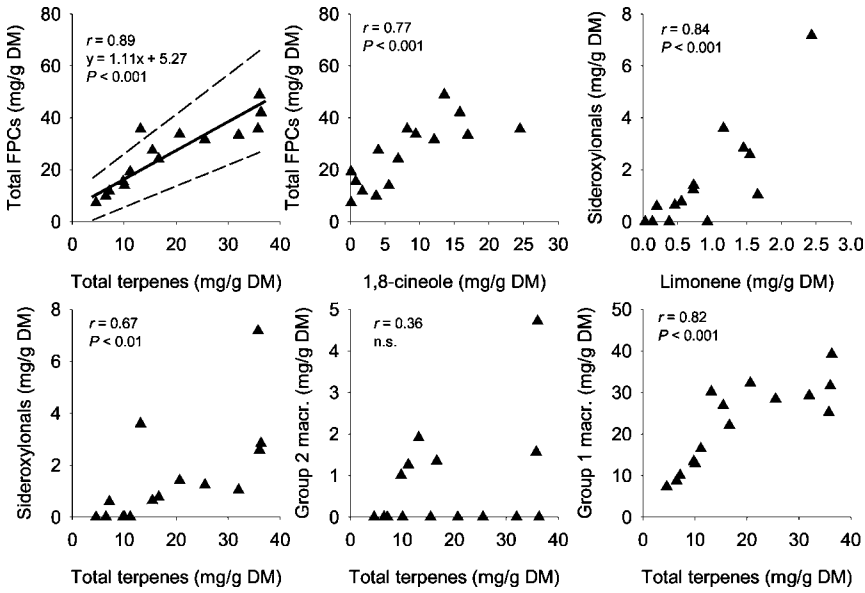

FIG. 10. Plotted FPC and terpene concentrations in 15 *E. viminalis* trees. Listed on each figure are the Pearson's correlation coefficient (r) and the associated significance level (P). The solid line indicates a major axis regression and the dashed lines indicate the 95% confidence interval.

folivores. In addition, the strong relationships between major terpene and FPC constituents may facilitate the development of conditioned flavor aversions that allow these animals to feed selectively. This study identified several previously unreported FPCs from *E. strzeleckii*, and for the first time described patterns of covariance amongst FPCs. This is a critical first step towards understanding the biosynthesis.

ESI-FTMS detected ion masses consistent with most known and several novel FPCs in our extracts, although jensenone (m/z 265.241) only occurred in one tree. One possible explanation of jensenone's rarity is that it is produced in these species, but only as a precursor of larger-molecular weight FPCs (Ghisalberti, 1996). The paucity of euglobals in our extracts is also surprising, given the large number of euglobal isomers previously reported from these species. ESI-FTMS indicated that flavones and cyclic polyketones, which differ from simple FPCs primarily in their functional groups, were generally only present in low concentrations. However, pentacyclic triterpenes such as ursolic acid produced strong ion peaks. The implications (if any) of these biologically active compounds for marsupial herbivores remain unknown.

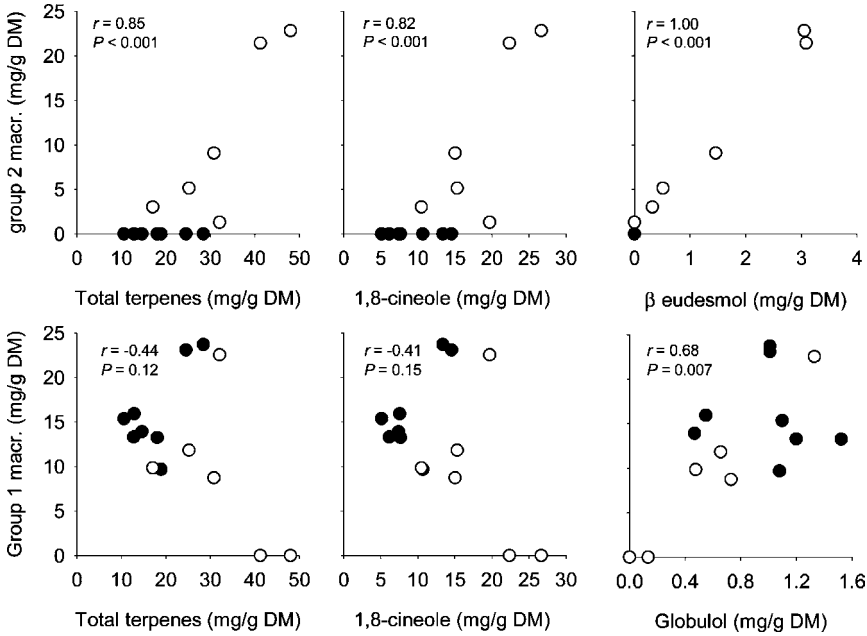

FIG. 11. Plotted FPC and terpene concentrations in 14 *E. globulus*, including trees possessing "group 2" macrocarpals (open circles) and trees without "group 2" macrocarpals (closed circles). Listed on each figure are Pearson's correlation coefficient (r) and the associated significance level (P), calculated across all 14 trees.

Like *E. melliodora* and *E. polyanthemos*, *E. strzeleckii* and the sideroxylonal-rich chemotype of *E. ovata* did not possess significant concentrations of most macrocarpal-type acylphloroglucinol-terpene adducts, including the sesterterpene and triterpene adducts proposed by Eyles et al. (2003). This suggests the possibility that a single enzyme may be responsible for combining acylphloroglucinol and terpenoid residues to produce a range of macrocarpals.

Several compounds present in *E. strzeleckii*, including jensenal, **7**, and **8**, differ from the more common dimeric acylphloroglucinols, such as sideroxylonals and grandinal, in that the 5-carbon acyl functions are not incorporated in the bonds forming the dimer. Consequently, these acyl functions may influence the effectiveness of these compounds as antifeedants. Hydrogen bonding of equivalent carbonyl groups with phenolic hydroxyl groups is essential to the action of grandinol as an inhibitor of both germination (Bolte et al., 1985) and activation of the Epstein-Barr virus (Takasaki et al., 1990). Jensenal, **5**, and **7** also differ from most FPC molecules in possessing only one formyl group compared to macrocarpals with two, and sideroxylonals with four. Although the formyl groups are thought

to be prerequisite to the antifeedant activity of FPCs (Lawler et al., 1999a), it is unclear as to whether their number is important.

In isolation, our mass spectrometry results imply that sideroxylonals were present only in trace amounts in *E. globulus* and *E. viminalis* and in lower concentrations than macrocarpals in most *E. ovata*. The only previous survey of FPC distributions used ESI-FTMS to assess the presence and the absence of known FPCs in 41 species of *Eucalyptus* (Eschler et al., 2000). In that study, sideroxylonals were the most frequently identified group of compounds overall, but were not detected in *E. globulus*. Our HPLC results indicated that sideroxylonals were present in substantial concentrations in many of our trees, including *E. globulus* and *E. ovata*. We showed that macrocarpal G produces a peak more than an order of magnitude greater than that from an equivalent molar concentration of sideroxylonal A. The most likely explanation of this difference is that macrocarpals are more readily ionized than sideroxylonal.

In most cases, the strongest terpene–FPC relationships involved specific monoterpenes, usually either 1,8-cineole or limonene. This pattern is well illustrated by *E. melliodora* and *E. polyanthemos*, in which sideroxylonal concentrations fell on the regression line predicted by 1,8-cineole and were not influenced by the high concentrations of other terpenes. There were strong correlations between terpene concentrations and each of the classes of FPCs when considered individually, but unsurprisingly these relationships were weaker for less dominant FPC classes. Correlations between FPC and monoterpene concentrations may result if the regulation of their synthetic enzymes is linked.

The relationship between "group 2" macrocarpals and α-, β-, and γ-eudesmol in *E. globulus* may be more direct because these sesquiterpenes and the terpene moiety attached to the epimeric macrocarpals I and J share a eudesmane-type skeleton (Osawa et al., 1996). Hence, the synthesis of these compounds may be limited by a common sesquiterpene precursor. In *E. globulus* trees that contain "group 2" macrocarpals, the concentrations of "group 1" macrocarpals are positively correlated with globulol and structurally similar terpenes, as well as with aromadendrene and its similar terpenes. Globulol is structurally identical to the terpene moiety of macrocarpals A and B, and aromadendrene matches the terpene moiety of macrocarpal G. These terpene moieties probably share a common origin in bicyclogermacrene (Ghisalberti, 1996). The fact that these correlations did not exist in trees without "group 2" macrocarpals suggests that the synthesis of "group 2" macrocarpals and related sesquiterpenes competes with the synthesis of "group 1" macrocarpals and related terpenes. Although the availability of specific sesquiterpene precursors may determine the type of macrocarpals produced, the positive correlations between terpene and total macrocarpal concentrations suggest that allocation to macrocarpal acylphloroglucinol precursors may be determined upstream from the formation of the final terpene adducts.

Our results confirm the possibility of dominant volatile monoterpenes acting as cues to folivores feeding on eucalypt species containing complex mixtures of FPCs. However, the relationship between terpene and FPC concentrations is species-specific. Major axis regression coefficients indicated that sideroxylonal concentrations in *E. melliodora* trees were typically more than twice those in *E. polyanthemos* trees with the same 1,8-cineole concentrations. Similarly, FPC concentrations in *E. globulus* were almost twice those seen in *E. viminalis* with similar terpene profiles. As terpene-rich species are not necessarily FPC-rich, folivores' feeding decisions must be informed by both the concentration of terpenes in the foliage and the tree species.

The headspace concentration of terpene over the leaf surface may vary according to weather conditions, light intensity, time of day, and leaf age (Zini et al., 2002), affecting the reliability with which folivores can gauge actual foliar concentrations by olfaction. However, qualitative differences in terpene composition should be consistently distinct. Hence, the distinctive terpene profiles of the two *E. melliodora* trees that possessed negligible amounts of cineole and sideroxylonal, but substantial amounts of *p*-cymene and spathulenol, could potentially provide a cue for a conditioned flavor preference. Folivores may find it easier to identify trees that smell different than to distinguish between similar "weak" and "strong" smells, and may be able to detect the presence of particular terpenes more reliably than the absence of others. Similarly, the correlation between eudesmol terpene concentrations and "group 2" macrocarpals in *E. globulus* potentially allows herbivores to discriminate among trees on the basis of the types of FPCs that are present. Although "group 1" and "group 2" macrocarpals do not appear to differ in their effectiveness as antifeedants for koalas (B. D. Moore, unpublished data), compositional cues may be important where the biological activity of compounds does differ. Even if the composition of hydrodistilled oils does not correspond to headspace terpene composition, similar between-tree qualitative differences can be expected. Several studies have found that eucalypt headspace volatiles are richer in sesquiterpenes, including compounds known to play infochemical roles in other plant genera, than are hydrodistilled oils (Betts, 2000; Zini et al., 2003).

Our results show striking variation in the absolute concentrations of FPCs, particularly in *E. viminalis* and *E. ovata*, including trees with almost no FPCs and trees in which FPCs accounted for more than 6% of the dry mass of foliage. Although high FPC concentrations deter folivore feeding (Lawler et al., 2000), this variation means that some less defended trees will usually be available to animals. In this context, the lesser degree of variation in *E. globulus* and *E. strzeleckii* is noteworthy. In both cases, even the least defended trees contained considerable concentrations of FPCs.

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BEHAVIORAL CHANGES IN WORKERS OF THE LEAF-CUTTING ANT *Atta sexdens rubropilosa* INDUCED BY CHEMICAL COMPONENTS OF *Eucalyptus maculata* LEAVES

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Abstract—The response of *Atta sexdens rubropilosa* Forel workers to essential oils, epicuticular wax and hexane, dichloromethane, ethyl acetate, and methanol extracts of *Eucalyptus maculata* was evaluated. Hexane extracts of *E. maculata* interfered with the recognition mechanism among workers. The main active compounds identified from this plant were the sesquiterpenes elemol and β-eudesmol. These compounds may be responsible for the resistance of this species to ant attack.

Key Words—*Atta sexdens rubropilosa*, *Eucalyptus maculata*, leaf-cutting ant, ant behavioral changes, sesquiterpenes.

INTRODUCTION

Leaf-cutting ants live in obligatory symbiosis with a basidiomycete fungus and they grow on fresh leaves harvested by workers. The fungus provides ants with nutrients (Martin et al., 1969) and enzymes (Boyd and Martin, 1975). The ants, in turn, supply the fungus with a variety of substrates (Weber, 1972) and stimulate symbiotic fungal growth (Martin et al., 1975). Leaf-cutting ant workers obtain a significant part of their nutrients from plant polysaccharide hydrolysis products produced by the action of extracellular enzymes released by the fungus (Silva et al., 2003).

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Leaf-cutting ants in the genera *Atta* and *Acromyrmex* are considered a threat to Brazilian agriculture (Wilson, 1980). They are the most severe pests in the more than 6 million ha of *Eucalyptus* plantations in Brazil (Siqueira, 1990; Della Lucia and Fowler, 1993). Although these ants are highly polyphagous, some plants escape their attack. This apparent resistance may be due to the presence of secondary metabolites such as tannins and terpenoids that are toxic to the ant-fungus symbiosis. According to Hubbell and Wiemer (1983), caryophyllene epoxide extracted from *Hymenaea courbaril* is toxic to the ant's own fungus and also repellent to the ants. Such double toxicity also occurs when leaf-cutting ants are fed daily with *Ricinus communis* (Hebling et al., 1996). Some active compounds such as *trans*-β-ocimene from *Astronium graveolens* (Chen et al., 1984) or the saponins from *Dioscorea cayenensis* (Febvay et al., 1985) act merely as ant repellents. Other plant compounds act as fungicides, e.g., sesamine (Bueno et al., 1990) and other unidentified compounds extracted from *Canavalia ensiformis* (Hebling et al., 2000). Anjos and Santana (1994) observed biting and mutilation of body parts by workers of *Atta laevigata* and of *Atta sexdens rubropilosa* exposed to leaves of four species of *Eucalyptus*, including *E. maculata*.

Our study aimed at isolating and identifying chemical components extracted from leaves of *E. maculata* that induce aggression in nestmates of *A. sexdens rubropilosa*. We also describe the behavior of ants exposed to different concentrations of the most active component.

METHODS AND MATERIALS

Biological Material. *E. maculata* leaves were collected during May (dry and winter season) from the middle of the tree canopy in a Eucalyptus stand at the campus of the Federal University of Viçosa. Leaves were placed onto ice and transported to the Chemistry Department of the Federal University of São Carlos, in São Paulo State, Brazil, where the leaf extracts were prepared.

Five colonies of *A. sexdens rubropilosa*, maintained in the laboratory for 2 years at $25 \pm 5^{\circ}$C, $75 \pm 5\%$ RH, and 12:12 (L:D), were used in our experiments. These colonies were housed in glass chambers interconnected by transparent hoses 3 cm in diameter and connected to a foraging arena (Della Lucia et al., 1993). Each colony had approximately 18 l of fungus, distributed in six chambers, along with abundant brood and thousands of workers. The colonies were supplied daily with water and leaves or flowers of several plant species such as *Ligustrum japonicum*, *Tecoma stans*, *Acalifa wilkesiana* and *Rosa* spp.

Extraction of Essential Oil and Epicuticular Wax. Essential oil was extracted from *E. maculata* leaves (400 g) by steam distillation for 4 hr in a Clevenger apparatus using 1 l water (German Pharmacopoeia), yielding 3.17 mg (0.79%). Epicuticular waxes were extracted from 60 fresh leaves immersed three times for 30 sec in CH_2Cl_2 at room temperature. The resulting solution was filtered

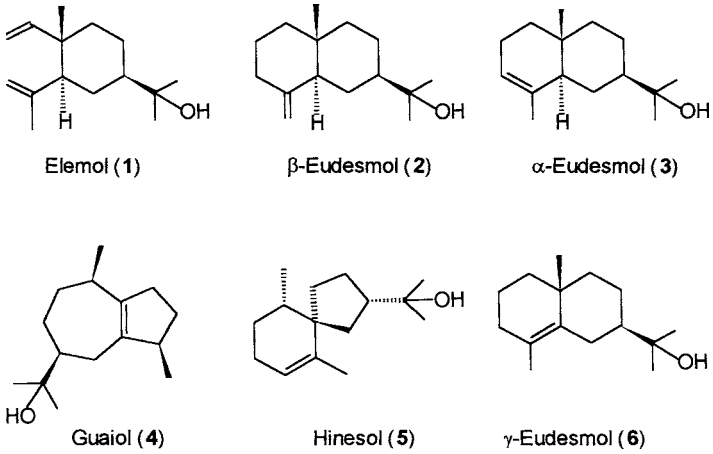

FIG. 1. Chemical structures of the sesquiterpenes identified in the most active fraction of the *E. maculata* leaf extract.

and dried; the solvent was removed under reduced pressure and yielded 190 mg (0.0812 mg/cm^2).

Chemical Analysis. The active sesquiterpenes (Figure 1) were extracted from *E. maculata* leaves (4.4 kg) with hexane (5 l), followed by chromatography on a silica gel flash column by elution with a gradient of hexane (8.3 g), CH_2Cl_2 (10 g), EtOAc (1.5 g), and methanol (0.2 g, 1 l of each solvent). The hexane and CH_2Cl_2 fractions were combined (18.3 g) and refractionated on silica gel, using CH_2Cl_2 as eluent, yielding four fractions (fraction 1, 1.3 g; 2, 1.7 g; 3, 3.2 g; and 4, 5.8 g). Compounds from fraction 4 (400 mg, mixture of compounds 1–6, Figure 1) were isolated, first by flash chromatography on silica gel yielding three fractions. Fraction 4-2 (279 mg) was fractionated by TLC on silica gel coated with 10% of $AgNO_3$ and eluted with Hex:EtOAc 7:3 to yield elemol (62 mg), β-eudesmol (63 mg), and mixture A (92 mg of α-eudesmol, β-eudesmol, guaiol epimer, γ-eudesmol, and hinesol).

The mixture of sesquiterpenes and pure compounds were analyzed on a Shimadzu QP 5000 mass detector running in the EI (electron impact ionization) scan mode, equipped with HP-1 fused-silica columns (25 m $\times$ 0.2 mm-ID, 0.11-μm film thickness); temperature program: 100°C for 1 min to 250°C at the rate of 5°C/min and 10 min at 250°C; splitless injector 225°C; detector 250°C. Spectra were obtained in electron impact mode at 70 eV.

Retention Index. Calculations of the Retention Index were performed using a Shimadzu 17A equipped with DB-5 fused-silica columns (30 m $\times$ 0.25 mm-ID, 0.25-μm film thickness); helium was used as the carrier gas at a constant flow rate of 0.9 ml/min; the oven temperature was held at 100°C for 1 min, increased at the

rate of 5°C/min to the final temperature of 250°C, and held for 10 min; injector 225°C and flame ionization detector (FID) 300°C. Calculation of retention indexes was achieved through coinjection with C_9–C_{22} *n*-hydrocarbons (van dan Dool and Kratz, 1963). Identification of the oil constituents was made on the basis of their retention indexes (Adams, 1995) and by comparison of mass spectra with the NIST library.

Bioassays. Bioassay 1 was conducted with the essential oil (4 mg/ml) and epicuticular wax (5 mg/ml), obtained from fresh leaves, as well as with hexane, dichloromethane, ethyl acetate, and methanol extracts (100 mg/ml), from air-dried leaves. Bioassay 2: fractions 1–4 of the hexane extract (10 mg/ml). Bioassay 3: subfractions 4-1 to 4-3 (8.5 mg/ml). Bioassay 4: pure compounds elemol and β-eudesmol or mixture A of sesquiterpenes (1 mg/ml). A total of four bioassays were carried out to guide the isolation of pure compounds. The refractionations were performed from the most active sample in the biological assays. Bioassays were conducted using five laboratory colonies of *A. sexdens rubropilosa*. These bioassays had the purpose of directing the fractionation necessary to isolate the active compounds. They were carried out as follows:

(1) Leaves used as substrates for the colonies were removed 6 hr before the tests were initiated. (2) Filter-paper fragments in the shape of either a rectangle, a square, or a triangle, each 6 cm^2 in area, were prepared as: (a) control, impregnated with the solvent (square); (b) blank (rectangle); and (c) the treatment to be assayed, using 100 μl of the solution of extract or pure compound (triangle). *A. sexdens* exhibits no preference among these shapes (Santana et al., 1990). (3) After solvent evaporation, two filter-paper fragments of each of the three different geometric shapes were placed on to three glass slides and then placed into the colony foraging arena at equal distances from the nest entrance. The positions of the three slides were periodically alternated to avoid learning. (4) Observations were made for 30 min after paper placement, by three observers who also alternated their position as to what to observe at each test (Santana et al., 1990). Number of aggression groups formed, the number of ants per group, and number of mutilated ants in each group were recorded. (5) A total of five replicates were performed per treatment in each of the five colonies of *A. sexdens rubropilosa*. The overall average was used in the analyses.

An aggression group was defined as two or more nestmates undergoing biting and mutilation, leading to death. These groups were collected and placed, individually, into 10-ml vials (2 cm in diam). Later, the number of aggression groups formed and the total number of mutilated ants per group in the 30-min observation period were counted.

An experiment with each of the five ant colonies was conducted to better describe the entire sequence of behavioral acts performed by workers of *A. sexdens rubropilosa* from the moment the paper fragment treated with the most active fraction of the leaf extract (1.0 mg/ml) was placed into the arena,

to the time when interaction ceased. The experiment was recorded on VHS to aid in the description and facilitate measuring the time spent performing various acts.

An additional experiment was designed to evaluate the effect of various concentrations of the most active fraction on the alteration of workers' behavior. Paper fragments were treated with increasing concentrations of β-eudesmol (0, 10, 100, 500, and 1000 μg/ml) corresponding to 0, 0.17, 1.67, 8.33, and 16.67 μg/cm^2. Three observations were conducted for each experimental concentration in each of the five colonies.

RESULTS AND DISCUSSION

Bioassays. All materials tested in Bioassay 1 showed some degree of bioactivity, with the exception of the epicuticular wax (Table 1). The hexane extract was the most active and further investigation was done using this extract for fractionation.

In Bioassay 2, most of the treatments showed some effect on worker behavior, with the exception of the ethyl acetate and methanol fractions. Fraction 4 showed

TABLE 1. NUMBER OF GROUPS FORMED AND NUMBER OF MUTILATED ANTS, ± STANDARD ERROR, 30 MIN AFTER THE ANTS WERE EXPOSED TO THE TREATMENTS DURING THE FOUR BIOASSAYS ($N = 5$)

Bioassay	Treatment	Number of groups	Number of mutilated ants
1	Epicuticular wax	0	0
	Essential oil	2.2 ± 1.5	3.6 ± 3.3
	Hexane extract	15.2 ± 5.6	21.6 ± 13.7
	CH_2Cl_2 extract	3.6 ± 2.7	7.0 ± 6.7
	EtOAc extract	6.8 ± 4.3	17.4 ± 14.6
	Methanol extract	3.8 ± 2.0	4.6 ± 3.0
2	Hexane 1	5.0 ± 4.3	6.6 ± 6.3
	Hexane 2	9.8 ± 6.5	18.4 ± 17.4
	CH_2Cl_2 3	10.0 ± 6.2	18.4 ± 16.9
	CH_2Cl_2 4	23.0 ± 11.1	111.8 ± 69.3
	Fraction EtOAc	0	0
	Fraction methanol	0	0
3	Subfraction 4-1	0	0
	Subfraction 4-2	42.8 ± 22.9	224.2 ± 144.9
	Subfraction 4-3	0	0
4	Elemol	47.4 ± 16.5	140.2 ± 72.2
	β-eudesmol	84.2 ± 13.2	285.8 ± 58.2
	Mixture A (α-eudesmol, β-eudesmol, guaiol epimer, γ-eudesmol, and hinesol)	25.2 ± 4.6	46.6 ± 9.0

larger numbers of groups and of mutilated ants than the entire hexane extract, even though this fraction was used at one tenth the concentration (10 vs. 100 mg/ml). Therefore, this fraction appeared to concentrate the compounds responsible for the alteration in behavior.

In Bioassay 3, we determined that fractions 4-1 and 4-3 failed to influence the ants. Fraction 4-2 was extremely active and was, therefore, selected for further fractionation. All subfractions tested in Bioassay 4 showed a high degree of activity.

The active fractions from *E. maculata* leaves were identified as the sesquiterpenes elemol and β-eudesmol, and the mixture α-eudesmol, β-eudesmol, guaiol epimer, γ-eudesmol, and hinesol. Identification of sesquiterpenes (**1–6**, Figure 1) was based on comparison of the reported mass spectrometry, retention index [elemol: 1550 and 1549; β-eudesmol: 1654 and 1649; α-eudesmol: 1655 and 1652; guaiol epimer: 1632 and 1595 (Guaiol); γ-eudesmol: 1634 and 1630; hinesol: 1642 and 1638, respectively for observed and literature (Adams, 1995)], and ^{1}H, and ^{13}CNMR data for elemol (Su et al., 1995), β-eudesmol (Jolad et al., 1988), and α-eudesmol (Chou et al., 1989).

Elemol and β-eudesmol were the most active ingredients in Bioassay 4; the latter compound produced the highest number of aggression groups and mutilated ants. β-Eudesmol, at 1 mg/ml, induced the formation of 5.5 times more groups than the hexane extract at the concentration of 100 mg/ml. The ratio between the numbers of mutilated ants for these two treatments was 13:2.

Our results demonstrate that the sesquiterpenes present in foliar extracts of *E. maculata* (viz. elemol and β-eudesmol) are the main compounds involved in this eucalyptus resistance. Some *Eucalyptus* species, such as *E. acmeniodes* and *E. torelliana* among others, are known to be more resistant to leaf-cutting ants than other species because they somehow interfere with foraging by workers (Anjos and Santana, 1994). *E. maculata* is also avoided by these ants in nature and, induces the aggressive behavior we observed.

Behavioral Sequence of A. sexdens rubropilosa *Workers After Exposure to β-Eudesmol.* Upon exposure, the ant touches the impregnated filter-paper with antennae, walks over the filter paper, and cleans antennae and tarsi with the mandibles. Time spent $= 23.70 \pm 1.84$ sec. The ant behaves as if alarmed: it displays open mandibles and is very excited. When an ant encounters a nestmate that had already been in contact with the paper, it touches the other with its antennae and then attacks, most frequently legs, but also other body parts. Time spent $= 8.30 \pm 0.68$ sec. The attacked worker then counter-attacks, and an aggression group is formed. Time spent $= 3.80 \pm 0.39$ sec. After formation of the first group, ants that are walking over the filter-paper near that group become excited, display open mandibles, and attack other ants, thus forming new groups; the number of ants on and around the papers increases. Time spent $= 29.24 \pm 0.03$ min. After 30 min of observation, a large number of aggression groups are seen on the arena

(with at least two ants in each), along with a large number of mutilated ants, missing legs, antennae, and abdomens.

Behavioral Response of A. sexdens rubropilosa *Workers to Different Concentrations of β-Eudesmol.* A linear dose-response in the number of aggression groups formed (Figure 2) and the number of mutilated ants (Figure 3) was observed. Behavioral modification of the leaf-cutter *A. cephalotes* was reported by Hubbell and Wiemer (1983) upon exposure to concentrations of caryophellene epoxide 10 times higher than normal. According to these authors, ants jumped or reared back into the arena and began intense grooming. Our results also demonstrated that higher order response of aggressive behavior correlates with concentration of β-eudesmol. The aggressive response suggests that the chemicals may interfere with nestmate recognition. Nestmate recognition has been widely studied (Carlin and Hölldobler, 1987; Lahav et al., 1999; Lenoir et al., 1999) and is thought to result from a mixture of endogenous (genetic) and exogenous (environment) factors to produce a Gestalt odor (Crozier and Dix, 1979). The relative importance of each of these factors varies with species, although little is known about it in leaf-cutting ants (Jutsum et al., 1979; Viana et al., 2001; Hernandez et al., 2002). β-Eudesmol might interfere with nestmate recognition by changing hydrocarbon profiles or by blocking olfaction. Elucidation of the mechanism at the physiological level could result in alternative control strategies against this pest species.

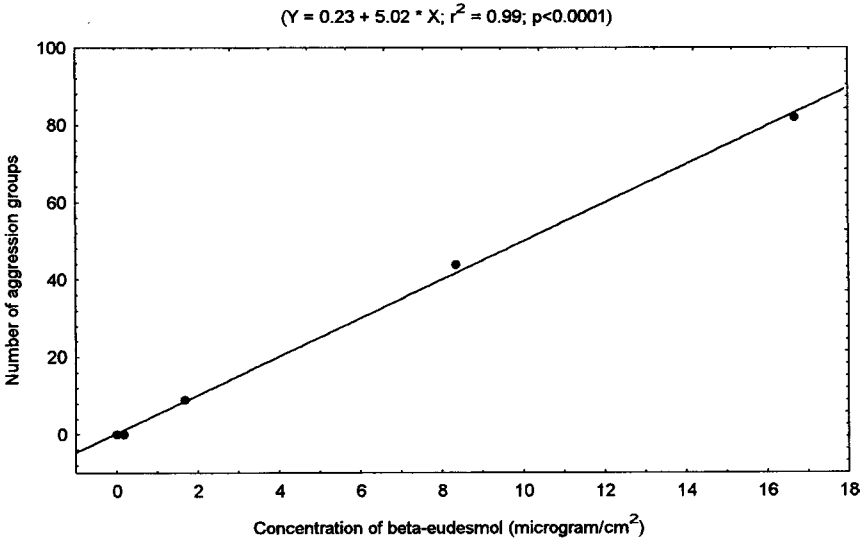

FIG. 2. Scatterplot and estimated values of the relationship between number of aggression groups and concentration of β-eudesmol.

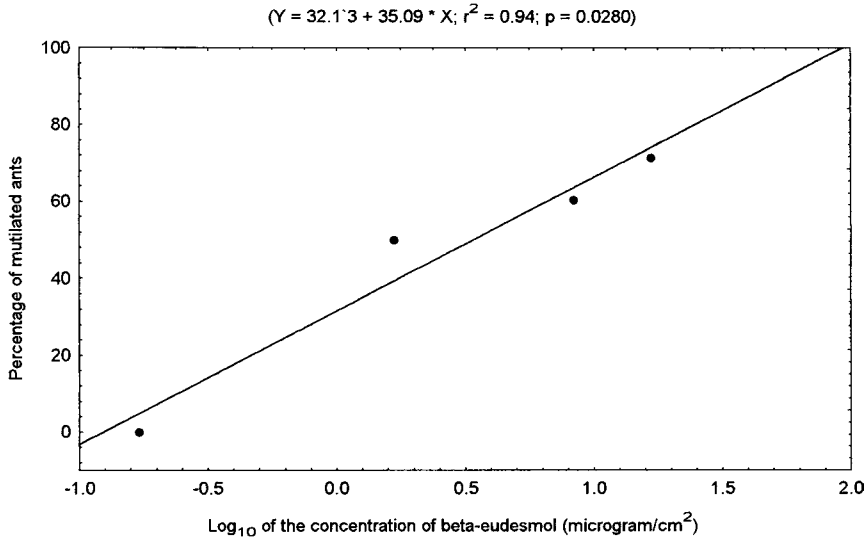

FIG. 3. Scatterplot and estimated values of the relationship between percentage of mutilated ants and the decimal logarithm of the concentration of β-eudesmol.

In summary, the sesquiterpenes elemol and β-eudesmol interfered with the mechanism of nestmate recognition among workers of *A. sexdens rubropilosa*; β-eudesmol was the most active. Exposed ants became aggressive. The presence of these compounds in *E. maculata* suggests that they could be responsible for resistance of this plant to the ant attack. A means for introducing this compound into the nest via granulated baits or thermofogging should be investigated, in an attempt to control ants, when they become a nuisance.

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ATTACK RATE AND SUCCESS OF THE PARASITOID *Diaeretiella rapae* ON SPECIALIST AND GENERALIST FEEDING APHIDS

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Abstract—*Lipaphis erysimi* (Kaltenbach) is a specialist crucifer feeding aphid and *Myzus persicae* (Sulzer) is a generalist feeding aphid. The foraging behavior of *Diaeretiella rapae* (McIntosh), a parasitoid with the ability to parasitize both of these species, was assessed using a series of attack rate and success bioassays, with turnip, *Brassica rapa* var *rapifera*, as the host plant. The attack rate of *D. rapae* was significantly greater on *L. erysimi* than on *M. persicae* when aphids were feeding on turnip leaf discs in Petri dishes, irrespective of the aphid species upon which the parasitoids were originally reared. Attack rate bioassays with leaf discs absent, using both satiated and starved aphids, revealed that background chemistry and internal aphid chemistry may have small effects on attack rate. Excision of *D. rapae* pupae from mummy cases and subsequent use of the fully developed adults in attack rate bioassays showed that cues received by *D. rapae* at the time of adult emergence provide cues that prime *D. rapae* to attack *L. erysimi* at a greater rate than *M. persicae*. However, the relative success of *D. rapae* on these two aphid species, in terms of the percentage of attacks resulting in a successful adult parasitoid, was not significantly different.

Key Words—Behavior, glucosinolate, *Myzus persicae*, *Lipaphis erysimi*, *Diaeretiella rapae*, Brassica, turnip, parasitoid, aphid.

INTRODUCTION

Plants of the Brassicaceae provide an ideal model for studying chemical interactions between plants and insects, including signalling to the third trophic level, due

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to their extensive and interesting secondary chemistry. A range of defensive glucosinolate molecules is included in this chemical composition and is of particular interest. Damage of plant tissue, including physical damage by herbivorous insects, facilitates the contact of glucosinolates with glucosinolate hydrolases, a group of enzymes generically known as myrosinases. The organization of the myrosinase–glucosinolate system has been reviewed by Bones and Rossiter (1996). Hydrolysis of glucosinolates results in the release of a number of volatile and nonvolatile products such as thiocyanates, nitriles, and, of particular interest, isothiocyanates (Larsen, 1981; Poulton and Moller, 1993) that have been implicated as important signalling molecules to insect parasitoids (Bradburne and Mithen, 2000).

The Aphididae and particularly the subfamily Aphidinae include major agricultural and horticultural pests and can be categorized as either specialist or generalist depending upon their host range. Specialization may occur to different degrees, such as the species or family levels. To feed on the Brassicaceae, aphids must be able to tolerate or utilize the toxic glucosinolates and their by-products. The specialist brassica feeding aphid, *Lipaphis erysimi*, has a host range limited to plants containing glucosinolates. *L. erysimi* sequesters ingested glucosinolates, the toxicity of which may provide defense against attack by generalist predators or parasitoids. Like the host plant, this aphid produces a myrosinase located in the head and thorax regions that hydrolyzes glucosinolates (MacGibbon and Beuzenberg, 1978; Jones et al., 2001). The peach–potato aphid *Myzus persicae* (Sulzer) (Homoptera: Aphididae) also colonizes brassicaceous plants, but uses a strategy of toleration, whereby glucosinolates and other plant secondary metabolites are flushed rapidly through the aphid gut and are present in the honeydew (Weber et al., 1986).

Diaeretiella rapae is regarded as a specialist parasitoid of brassica feeding aphids because of its ability to parasitize a range of aphids feeding on brassicas where it frequently occurs, although it is considered to have a potential host range of greater than 60 different aphid species (Pike et al., 1999).

Host selection by parasitoids has been divided into numerous steps but we will adopt the widely accepted ideas of Vinson (1985), who subdivided host selection into five steps: Host habitat location, host location, host acceptance, host suitability, and host regulation.

Successful parasitism involves not only the location of a host, but an oviposition and subsequent development and emergence of an adult parasitoid from a mummified aphid. We hypothesized that the differentially adapted aphids used in this investigation would differ in their suitability as hosts to *D. rapae*, and that host acceptance would be influenced as a result. The differential attack rates of *D. rapae* on *L. erysimi* and *M. persicae*, and the success of attacks on these aphid species, were investigated. The effect of host aphid species, and how cues encountered upon emergence from mummy cases may condition future parasitoid behavior, was also examined.

METHODS AND MATERIALS

Plants. The plants used in all experiments and in all insect cultures were turnip *Brassica rapa* var rapifera of the cultivar Tokyo Cross F1 (supplied by EW King, Monks Farm, Essex, U.K.). All plants were grown at Rothamsted Research in greenhouses, in 1-l plastic pots containing a compost mix prepared by Petersfield Products, Cosby, Leicester, U.K. Maximum temperature and light depended upon season and time of day, but minimum light was maintained at 14 hr/d using Sodium High Pressure Lamps. Minimum temperature was maintained between 18 and 25°C.

Insects. Cultures of *L. erysimi* (Kaltenbach) on turnip were started from stocks reared on Chinese cabbage at Rothamsted Research. *M. persicae* (Sulzer) cultures on turnip were started from *M. persicae* stocks reared on oilseed rape (*Brassica napus*). These "new" aphid cultures were cycled through at least six generations on the turnip plants before parasitoid cultures were started. *D. rapae* (McIntosh), originally from stocks at Rothamsted Research reared on *M. persicae* on Chinese cabbage, were established as separate cultures on *M. persicae* and *L. erysimi*, both on turnip. The new aphid and parasitoid stocks were kept in controlled environment rooms with a temperature of 23°C and a photoperiod of 16L, 8D. At least six generations of *D. rapae* were cycled through the aphid–turnip system before experiments were started.

Aphids used in experiments were removed directly from leaves of stock cultures with a fine paintbrush and transferred to Petri dishes. Second and third instar nymphs were used in all Petri dish experiments. Single aphids were crushed using forceps, which resulted in the release of alarm pheromone and agitated the surrounding, previously static, aphids. The agitated aphids removed their stylets and were then transferred without damage; 99% of *M. persicae* aphids of the standard laboratory clone US1L move in response to synthetic aphid alarm pheromone (Dawson et al., 1983), and 99% of *L. erysimi* move in response to crushed aphid extract (Dawson et al., 1987).

D. rapae were removed from turnip plants as mummified aphids using watchmaker forceps to pry away the mummy at the point where it adjoins the leaf surface. Mummies were stored in darkened plastic containers leading into glass jars. Newly emerged adult parasitoids congregated in the jars, as they display positive phototaxis. At 24-hr intervals, the glass jars containing parasitoids were removed and screwed onto a lid supporting a cotton wool ball soaked with 25% honey solution as a food source for the parasitoids. At this time, a clean jar was affixed to the plastic container. This procedure was repeated until all adult parasitoids had emerged. The parasitoids collected in the glass jars were left for 3 d so that all females used in experiments should have been standardized mated females, with no adult experience of aphids or plants. All experiments were conducted at 21°C under a laminar light source.

Attack Rate Arenas. For attack rate bioassays involving plant material, arenas consisted of 8.5-cm diameter plastic Sterilin Petri dishes (Bibby Sterilin Ltd., Staffordshire, U.K.) containing a 7.3-cm diameter leaf disc embedded in 1.5% agar (Sigma, Poole, U.K.). The leaf discs were cut from the four turnip leaves in a whorl immediately within the outer two leaves using a circular cutter, one disc from each leaf. A total of 20 aphids, either *M. persicae* or *L. erysimi*, at the second or third instar stage were counted onto each leaf disc.

For bioassays without plant material, Sterilin Petri dishes with a 4.8-cm diameter were used. The sides of the Petri dishes were coated with fluon (ICI Ltd., Welwyn Garden City, Herts, U.K.), which was applied using cotton wool. A total of 10-sec or third instar *M. persicae* or *L. erysimi* were counted into each arena. The presence of fluon prevented the dispersal of the aphids up the sides and across the tops of the arenas. The aim of this was to minimize aphid dispersal rates and reduce the effect that differential dispersal rate and aphid velocity would have on the results of these bioassays. The smaller (4.8 cm diameter) Petri dishes were used in these experiments so that bioassays could be more easily recorded onto VHS cassettes *via* a video camera linked to a VHS recorder and monitor.

Effects of Parasitoid Age on Attack Rate. A preliminary experiment was conducted to observe the effects of parasitoid age on attack rates. Individual *D. rapae*, reared on *L. erysimi*, were added to an arena containing aphids on a leaf disc and observed for 15 min. The number of attacks, defined as a clear contact between the parasitoid ovipositor and an aphid, and the time of the first attack were recorded. A total of 16 replicates were made for newly emerged and 1-, 2-, 3-, 4-, and 5-d-old parasitoids. On the basis of the results, 3-d-old parasitoids were used in all subsequent bioassays.

Attack Rate Bioassays. Individual female *D. rapae* were added to arenas containing aphids on leaf discs, and the number of attacks made in 15 min was recorded. Thirty-eight replicates were made for each *D. rapae* (reared on *M. persicae* or *L. erysimi*) host combination.

A second set of bioassays was conducted in arenas without leaf discs. Some of these bioassays were recorded onto VHS cassettes and analyzed at a later time, thus allowing more replicates to be completed on a single day. Fifty-two replicates were completed with *D. rapae* reared on *L. erysimi*, and 49 replicates with *D. rapae* reared on *M. persicae*.

Bioassays utilizing starved aphids were also conducted in arenas without leaf discs. The aphids used for these were removed from stock cultures with a fine paintbrush 16 hr prior to the start of the bioassays and kept in glass specimen jars. This gave the aphids sufficient time in which to metabolize the vast majority of accumulated plant secondary compounds and allowed a comparison of the two hosts with plant secondary chemistry eliminated as a cue during parasitoid host selection. Female *D. rapae* were added individually to arenas and observed for

15 min. Some of the bioassays were recorded onto VHS cassettes for analysis at a later time. Fifty-three replicates were completed with *D. rapae* reared on *L. erysimi* and 40 replicates with *D. rapae* reared on *M. persicae*.

In a further bioassay, *D. rapae* were collected from stock cultures as mummies, but they were excised from their cases prior to adult eclosion. Excision involved carefully tearing apart the basal area of the mummy case, where it was attached to the leaf surface, by using watchmaker forceps. The immature parasitoids were placed onto Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, U.K.) moistened with 1% saline solution in glass Petri dishes. As the parasitoids completed adult development, they were removed from the Petri dishes and fed with 25% honey solution soaked into cotton wool balls. *D. rapae* were excised from both *L. erysimi* and *M. persicae* mummies. These parasitoids were then used in attack rate bioassays by using arenas with aphids and leaf discs, as described above.

A final set of attack rate bioassays was conducted using *D. rapae* that had been excised from their mummy cases, and subsequently placed within either a mummy case of the same host species on which it had developed ("home" experience) or a mummy case derived from the alternative aphid species ("away" experience). This allowed the parasitoids to touch the mummy cases with their antennae following the completion of adult development. Between 27 and 30 replicates were completed for each bioassay utilizing excised parasitoids.

Attack Success Bioassays. The proportion of attacks by *D. rapae* resulting in production of a larva was assessed. Five, second instar aphids, either *M. persicae* or *L. erysimi*, were introduced one at a time to individual female parasitoids, isolated in glass vials. Each aphid was removed after an attack had been made and each was kept on leaf material for 3 d after which they were carefully dissected using very fine probes in a drop of acetocarmine, a stain used for visualizing larvae. The number of aphids containing a parasitoid larva was recorded. *D. rapae* reared on *M. persicae* ($N = 25$) and *L. erysimi* ($N = 20$) were tested using both aphid hosts.

To assess the proportion of attacks by *D. rapae* resulting in production of a mummy, aphids were exposed to parasitoids in the same way as above. However, instead of dissecting the aphids after 3 d they were kept on leaf material until mummies formed (approximately 10 d). The number of mummies and intact aphids were counted, with mummies produced expressed as a percentage of the total number of aphids recovered. *D. rapae* reared on *M. persicae* ($N = 25$) and *L. erysimi* ($N = 12$) were tested using both aphid hosts.

Performance of *D. rapae* on the two aphid hosts was further assessed by determining the number of mummies produced by a single female parasitoid in a 24-hr period and the sex ratio and longevity of the resulting adults. Approximately 250 aphids, either *M. persicae* or *L. erysimi*, of mixed instars were added to a

single large turnip leaf in a cage. A single female parasitoid was added to this cage and left for 24 hr to attack the aphids. After 24 hr, the parasitoid was removed, and the aphids were left on the leaf to develop through to the mummy stage, at which time the mummies were removed from the plant and kept in a plastic container to emerge. The emergent parasitoids were sexed and fed with honey solution on paper twists. The longevity of these parasitoids was recorded. A total of 10 replicates were obtained for *D. rapae* reared on *L. erysimi* and *M. persicae* attacking each host species. Random samples of *D. rapae* were also taken from stock cultures reared on *M. persicae* and *L. erysimi* on turnip, and their mean sizes were compared by measuring the hind tibia lengths of males and females, using a microscope with 40× magnification and a graticule with graduations representing 0.017 mm.

Statistical Analysis. Student's t tests were used to analyze differences in *D. rapae* attack rate. Bioassays not resulting in any attacks were removed from the data set before analysis. Data from the attack success experiments were analyzed using a combination of Student's t tests and chi-square tests with Yates' correction for dealing with two categories.

RESULTS

Effects of Parasitoid Age on Attack Rate. Newly emerged *D. rapae* had a significantly lower attack rate than those aged 1, 2, 3, 4, and 5-d ($P < 0.01$). There were no significant differences in attack rate between parasitoids aged 1–5 d ($P > 0.05$) (Figure 1). Only 25% of newly emerged parasitoids, compared with at least 87.5% of parasitoids aged 1–5 d, made one or more attacks. The time taken by *D. rapae* to make an initial attack increased significantly after 4 d (Figure 2).

Attack Rate Bioassays. When attack rate bioassays were conducted in Petri dishes with leaf material present, *D. rapae* attacked *L. erysimi* at a greater rate than *M. persicae*, irrespective of which aphid it was reared on ($P < 0.01$). When leaf material was absent, *D. rapae* reared on *L. erysimi* did not have a greater attack rate on *L. erysimi* ($t = 0.99$; $P > 0.05$; df = 69). However, when reared on *M. persicae*, greater attack rate on *L. erysimi* was observed ($t = 3.07$; $P < 0.01$; df = 69). When the aphids were starved, *D. rapae* reared on *L. erysimi* had a greater attack rate on *L. erysimi* ($t = 2.53$; $P < 0.01$; df = 86), but those reared on *M. persicae* showed no significant difference ($t = 0.33$; $P > 0.05$; df = 54) (Figure 3).

There was no difference in attack rate on *L. erysimi* or *M. persicae* by *D. rapae* excised from *L. erysimi* mummy cases ($t = 1.25$; $P > 0.05$; df = 45). However, these parasitoids attacked *L. erysimi* at greater rates than *M. persicae* when allowed contact with either a *L. erysimi* mummy case ($t = 2.32$; $P < 0.05$; df = 56) or a *M. persicae* mummy case ($t = 2.70$; $P < 0.05$; df = 56) after emergence.

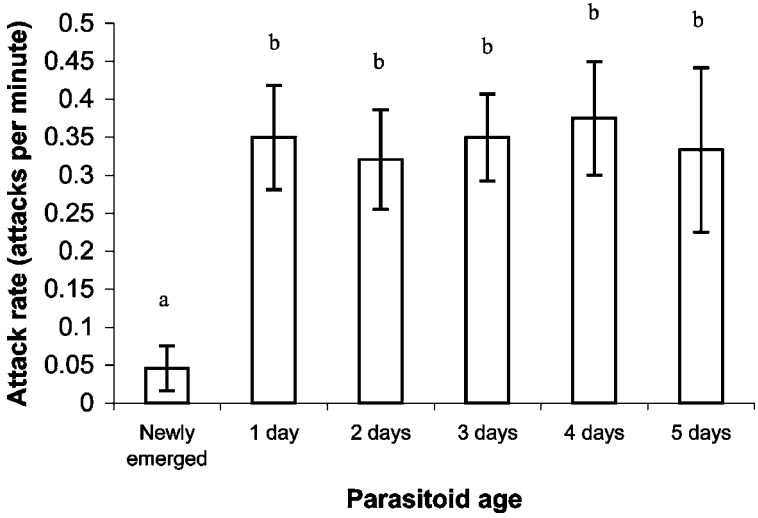

FIG. 1. Effect of age on attack rate of *D. rapae* females against *L. erysimi* on turnip leaf discs in petri dish bioassays lasting 15 min ($N = 16$). Different letters above the columns indicate a significant difference ($P < 0.05$).

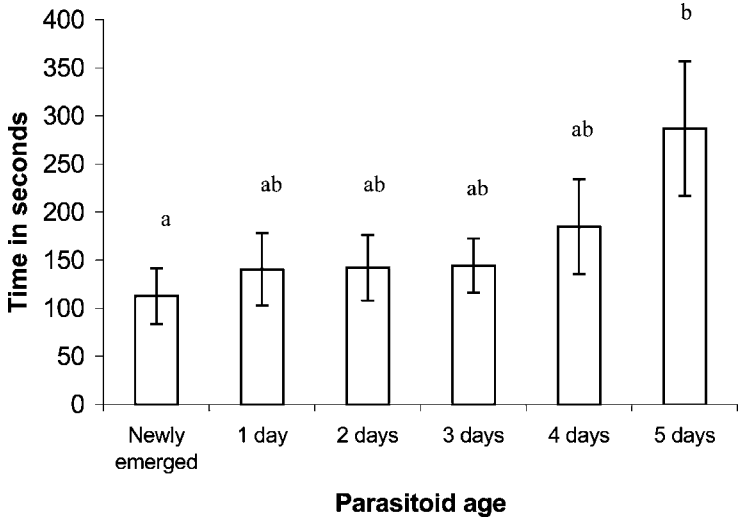

FIG. 2. Effect of age on time taken by *D. rapae* females to make an initial, full ovipositional attack on *L. erysimi*. Bioassays lasted for 15 min ($N = 16$). Different letters above the columns indicate a significant difference ($P < 0.05$).

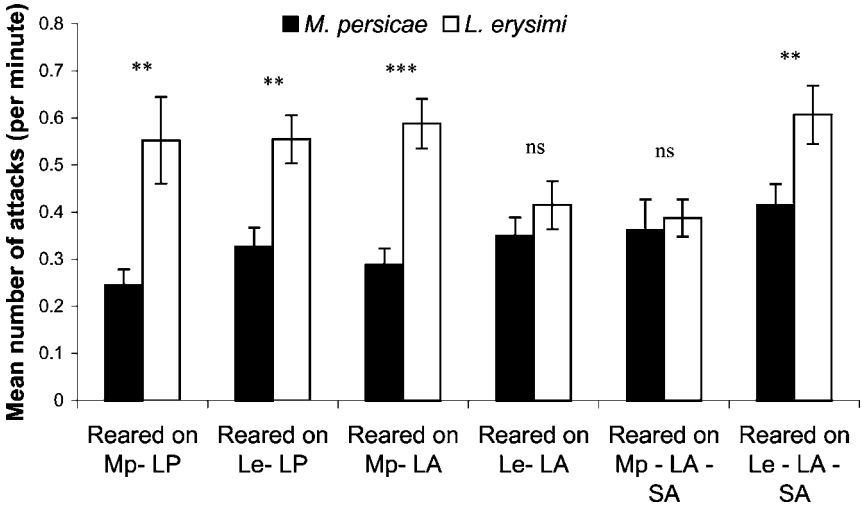

FIG. 3. Attack rate by *D. rapae*, reared on either *L. erysimi* (Le) or *M. persicae* (Mp), in arenas with leaf discs present (LP) or absent (LA) and with aphids that had been feeding directly prior to and/or during bioassays or starved for 16 hr prior to bioassays (SA). *$(0.05 > P > 0.01)$; **$(0.01 > P > 0.001)$; ***$(P < 0.001)$; ns = not significant.

D. rapae excised from *M. persicae* also attacked the two aphid species at the same rate ($t = 1.28$; $P < 0.05$; df = 56) (Figure 4) and attacked *L. erysimi* at a greater rate when allowed contact with either *L. erysimi* ($t = 5.03$; $P < 0.01$; df = 50) or *M. persicae* ($t = 2.07$; $P < 0.05$; df = 53) mummy cases.

Attack Success. The first five attacks made by *D. rapae* revealed no significant difference in the percentage of attacks resulting in larvae between the two aphid species, irrespective of the aphid species on which the parasitoids were reared. The percentage of the first five attacks by *D. rapae* resulting in a mummy was not significantly different from the number of attacks resulting in larvae (Table 1).

Individual *D. rapae* parasitoids foraging for 24 hr on either *M. persicae* or *L. erysimi* in small cages containing an excess of aphids produced a greater number of mummies on *M. persicae* than *L. erysimi*, irrespective of whether they were originally reared on *L. erysimi* ($t = 2.13$; $P < 0.05$; df = 18) or *M. persicae* ($t = 3.45$; $P < 0.01$; df = 18). The percentage of mummies resulting in an adult was not significantly different for the two host species. Parasitoids reared on *M. persicae* attacking *M. persicae* produced offspring with a significant male bias ($\chi^2 = 8.49$; $P < 0.01$) but no other sex ratios differed significantly from 1:1. The longevity of the adult parasitoids was between 4 and 10 d when emerging from either aphid species (Table 2).

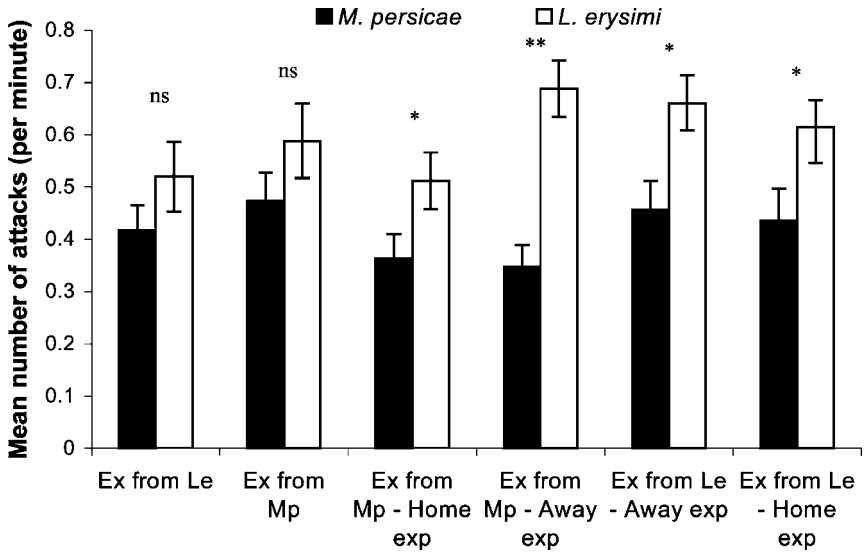

FIG. 4. Attack rate by *D. rapae* reared on *L. erysimi* (Le) or *M. persicae* (Mp) and excised (Ex) from mummy cases prior to adult eclosion. Some parasitoids were given a post-emergence experience of a "home" (the same aphid species) or "away" (the alternative aphid species) mummy case. *$(0.05 > P > 0.01)$; **$(0.01 > P > 0.001)$; ns = not significant.

The hind tibiae of male *D. rapae* reared on *L. erysimi* were longer than those of *D. rapae* reared on *M. persicae* ($t = 2.07$; $P < 0.01$; df = 38). The hind tibiae of female *D. rapae* reared on *L. erysimi* and *M. persicae* did not significantly differ in length ($t = 1.58$; $P > 0.05$; df = 31) (Table 3).

DISCUSSION

It was hypothesized that the foraging behavior of *D. rapae* would be influenced by the differential chemistry between the specialist aphid *L. erysimi* and the generalist *M. persicae*. Bioassays examining the attack rate of naïve *D. rapae* on these aphid species showed a greater attack rate on *L. erysimi* than on *M. persicae*, irrespective of the aphid species upon which the parasitoids were originally reared. This suggests that following emergence, *D. rapae* has a greater preference for *L. erysimi* than for *M. persicae*. This contrasts with past work using the parasitoid *Aphidius ervi*, in which host aphids used in the original rearing were always attacked at greater rates than alternative aphids (Pennacchio et al., 1994).

Attempts were made to ascertain why this preference exists, with a focus on the possible influence of plant chemistry, aphid chemistry, and/or the effects of conditioning during emergence from mummy cases. The influence of plant

TABLE 1. REPRODUCTIVE SUCCESS OF *D. rapae* FEMALES WHEN PRESENTED WITH INDIVIDUAL APHIDS TO ATTACK IN GLASS VIALS

Host *D. rapae* reared on	Aphid species attacked	Number of attacks resulting in a larve (N = parasitoids used)	Percentage of attacks resulting in a larva	Number of attacks resulting in a mummy (N = parasitoids used)	Percentage of attacks resulting in a mummy
M. persicae	*L. erysimi*	39[a]	37.1	41[a]	37.6
	M. persicae	46[a]	38.0	49[a]	40.5
L. erysimi	*L. erysimi*	33[b]	34.4	12[c]	30.8
	M. persicae	27[b]	34.2	20[c]	38.5

Note. Each female was presented with five aphids one at a time. The superscript letters indicate the number of parasitoids used for each parasitoid host combination $^{a} = 25, ^{b} = 20, ^{c} = 12$, percentages were calculated from the number of aphids recovered, and a small number of aphids were unaccounted for. There were no significant differences in the number, or percentage, of successful attacks.

TABLE 2. PERFORMANCE OF *D. rapae* FEMALES WHEN PROVIDED WITH AN EXCESS OF HOSTS FOR 24 HR IN A SMALL CAGE

Host *D. rapae* reared on	Aphid species attacked	Mean number of mummies	Percentage of mummies resulting in an adult	Sex ratio		Mean longevity	
				♀	♂	♀	♂
M. persicae	*M. persicae*	50.8[a]	78.0	169	227[b]	6.8	6.5
	L. erysimi	24.6	76.0	99	98	6.1	6.5
L. erysimi	*M. persicae*	41.8[c]	73.9	161	158	7.0	6.5
	L. erysimi	30.8	72.1	111	111	6.3	6.4

Note. $N = 10$ for each parasitoid–host combination. Significantly greater numbers of mummies were produced on *M. persicae* than *L. erysimi*, this is denoted by [a] $= P < 0.01$ and [c] $= P < 0.05$. No other significant differences were present. [b] denotes a significant difference in sex ratio ($\chi^2 = 8.49$; $P < 0.01$).

TABLE 3. THE MEAN HIND TIBIA LENGTHS OF MALE AND FEMALE *D. rapae* REARED ON *M. persicae* AND *L. erysimi*

	Mean hind tibia length of *D. rapae* (mm ± SE)	
Host aphid	♂	♀
M. persicae	0.49 ± 0.01^a ($N = 18$)	0.51 ± 0.01 ($N = 17$)
L. erysimi	0.52 ± 0.009^b ($N = 22$)	0.53 ± 0.007 ($N = 16$)

Note. Male *D. rapae* reared on *M. persicae* have significantly shorter hind tibias than *D. rapae* reared on *L. erysimi* ($t = 2.07$; $P < 0.05$), the superscript indicators indicate significant differences. Hind tibias of female *D. rapae*, reared on *L. erysimi* and *M. persicae*, did not significantly differ in length.

chemistry was investigated by using attack rate arenas minus leaf discs, which also eliminated a source of food for the aphids, and hence encouraged a greater amount of aphid activity. *D. rapae* reared on *M. persicae* had a significantly greater attack rate on *L. erysimi*, but *D. rapae* reared on *L. erysimi* showed no significant attack rate differences. The plant chemistry may have some influence on differential attack rate, but it is probably a minor effect.

Both aphid species were starved for 16 hr in an attempt to eliminate plant chemistry as a factor in parasitoid attack rate. In these bioassays, *D. rapae* reared on *L. erysimi* had a greater attack rate on *L. erysimi*, but *D. rapae* reared on *M. persicae* showed no significant differences in attack rate between the two hosts. Starving the aphids was assumed to remove glucosinolate content of aphids as a factor, but it is possible that this assumption was wrong and that aphid glucosinolate content was concentrated because of extreme dehydration of the aphids. Current work is focussing on finding out differences in glucosinolate content of starved and satiated aphids.

The third method used to elucidate why naïve *D. rapae* have a greater attack rate on *L. erysimi* than *M. persicae* was to remove cues encountered by parasitoids as they emerge from mummy cases (Storeck et al., 2000). Parasitoid pupae were excised from mummies and reared through to adulthood in Petri dishes, a technique pioneered by van Emden (see van Emden et al., 1996). Excised parasitoids showed no significant differences in attack rate irrespective of the aphid species upon which they were reared. It is proposed that there are chemical cues, probably glucosinolates, on the mummy case that are vital in priming the response of *D. rapae* to aphids, and that these priming cues are present on both *L. erysimi* and *M. persicae* mummy cases. Placing excised pupae within mummy cases so that the parasitoids will receive an experience as soon as they complete development confirmed that this early conditioning is vital and showed that either aphid species mummy case is equally suitable in terms of providing a priming cue when leaf discs are present. Storeck et al. (2000) showed the importance of cues received at

the time of emergence on the response of *Aphidius colemani* to long distance plant derived cues which, coupled with the information here, suggests that this early conditioning at the time of emergence is important in several aspects of parasitoid foraging behavior. We know that glucosinolates are present in the aphid carcasses and in the honeydew, but analysis of the glucosinolate content of mummy cases needs to be performed to confirm the importance of these chemicals in priming behavior in this instance.

It is possible that the priming cues received from the different mummy cases give varying degrees of priming resulting in parasitoids that display varying degrees of glucosinolate response depending upon their aphid host. *D. rapae* reared on *L. erysimi* develop in a background of high glucosinolate concentration, while *M. persicae* provides a relatively low glucosinolate environment, this may influence the sensitivity of *D. rapae* to glucosinolate cues encountered on the surface of potential host aphids. This, together with information on the glucosinolate content of starved aphids, will aid explanation of all the attack rate experiments. The important message from this work is that *D. rapae* emerging from both *L. erysimi* and *M. persicae* mummies will always be primed to preferentially attack *L. erysimi*, a behavior conditioned by cues received from the mummy case at the time of emergence.

Once an aphid attack has been made, it may or may not result in successful oviposition. Chemoreceptors on the parasitoid ovipositor allow an internal assessment of aphid quality, so an observed attack does not necessarily result in egg release. Therefore, differential egg release could indicate that *L. erysimi* and *M. persicae* differ in their quality as aphid hosts.

Attack success can be broken down into stages with the percentage of attacks resulting in egg deposition, formation of larvae, formation of mummies, and adult emergence all being measures of success. Egg deposition was not recorded here because of the potential inaccuracies associated with trying to locate parasitoid eggs, but the percentage of attacks resulting in larvae or mummies in *L. erysimi* and *M. persicae* did not differ significantly. Also, there were no differences in the number of attacks resulting in larvae and those resulting in mummies, suggesting that there is low mortality at the larval stage and that most larvae will successfully pupate and form mummies. Thus, neither aphid appears to be a better host for *D. rapae* in terms of the number of larvae or mummies that are formed following attack.

When parasitoids, reared on either *L. erysimi* or *M. persicae*, were exposed to aphids for 24 hr, a greater number of mummies were produced in the cages containing *M. persicae* than *L. erysimi*. This does not correlate with the results of the attack rate, and success bioassays that showed *L. erysimi* to be attacked at a greater rate than *M. persicae* and successful oviposition not to differ between the two hosts. However, it is important to note that the parasitoids were exposed to hosts for a much shorter time in these earlier bioassays. It is likely that differences

between the two aphid species other than chemistry are important in determining success, for example colonies of *L. erysimi* may be disrupted to a greater extent than *M. persicae* resulting in a greater number of aphid deaths prior to mummification due to aphids falling from the plant material. The longevity of parasitoid offspring did not significantly differ between those emerging from the two hosts, and in most cases the sex ratio was 1:1, which is in part contradictory to work done by Wilson and Lambdin (1987), who found the sex ratio to be skewed in favor of females, especially for 3-d-old *D. rapae* parasitoids. However, this may be due to differences in rearing conditions and genetic variability between the populations. One divergence from this 1:1 sex ratio occurred when *D. rapae* reared on *M. persicae* were exposed to *M. persicae*. In this case, the sex ratio was skewed towards males, suggesting that parasitoids reared on and attacking *M. persicae* may perceive *M. persicae* as a lower quality host and may not fertilize eggs accordingly.

Hind tibia length is a widely accepted measurement of parasitoid size. Female *D. rapae* reared on *L. erysimi* and *M. persicae* did not significantly differ in their hind tibia lengths. As parasitoid size is influenced by the size of the aphid, it is likely that *D. rapae* select similar-sized *L. erysimi* and *M. persicae* for depositing fertilized and select larger aphids for females than for males. The hind tibias of male *D. rapae* reared on *L. erysimi* were significantly longer than those reared on *M. persicae*, although the actual difference in lengths was small. When *D. rapae* assess the host quality of *M. persicae*, they may accept smaller aphids for depositing male eggs than they would accept when assessing *L. erysimi*. An excess of smaller *M. persicae* aphids may have been responsible for the drift from a 1:1 sex ratio observed.

Overall, *D. rapae* had a greater attack rate on the specialist crucifer feeding *L. erysimi* than on the generalist *M. persicae*, a behavior that is primed at the time of emergence from mummy cases. There are few significant differences in parasitoid success on these two host species, with a skew in sex ratio towards males when the parasitoids are reared on and are attacking *M. persicae*, plus significantly smaller males on *M. persicae*. The location by *D. rapae* of *M. persicae* and *L. erysimi* infested turnip has been investigated in a series of Y-tube bioassays, with no differences in attractiveness discovered (J. Blande, unpublished data). Combining this information with the greater attack rate on *L. erysimi* and the near equal success led us to predict that more *L. erysimi* mummies would be produced in small scale laboratory studies that allow *D. rapae* a 24-hr foraging period. The observations show the opposite to this, highlighting the need to consider a range of increasing experimental scales and methods such as simulation experiments and field trials to make predictions about the evolution and ecology of parasitoid–aphid relationships, especially if we wish to manipulate parasitoids in programs of biocontrol or integrated pest management.

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CORN PLANTS TREATED WITH JASMONIC ACID ATTRACT MORE SPECIALIST PARASITOIDS, THEREBY INCREASING PARASITIZATION OF THE COMMON ARMYWORM

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Abstract—We investigated whether corn plants treated with jasmonic acid (JA) increases the ability of the parasitic wasp, *Cotesia kariyai*, to find and control the common armyworm (*Mythimna separata*) under laboratory conditions. The rank order of attractiveness increased from intact plants treated with distilled water (DW) (2 d), JA-treated intact plants (2 d), DW-treated infested plants (2 d) to JA-treated infested plants (2 d). Single JA-treatment to either infested or uninfested plants increased attractiveness to *C. kariyai* over a period lasting at least 10 d. We then showed that the increase in attractiveness of infested corn plants by JA-treatment resulted in increased parasitism by *C. kariyai*. These results hold a promise for field application of JA-treatment. First, JA-treatment not only promotes the attractiveness of uninfested plants, but also armyworm-infested plants. Thus, parasitoids are not likely to waste time on JA-treated uninfested plants when JA-treated infested plants are available. Second, the effect of JA-treatment is lasting for at least 10 d, a result now obtained in two independent studies.

Key Words—Herbivore-induced plant volatiles (HIPV), jasmonic acid, parasitic wasp, *Cotesia kariyai*, common armyworm, *Mythimna separata*, corn plants, *Zea mays*, parasitization rate.

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INTRODUCTION

Plants damaged by herbivorous arthropods emit herbivore-induced plant volatiles (HIPV) that attract carnivorous natural enemies of herbivores (e.g., Takabayashi and Dicke, 1996; Sabelis et al., 1999; Dicke, 2000). The mechanisms leading to the production of HIPV by plants have received considerable attention from chemical ecologists and molecular biologists. Jasmonic acid (JA) is one of the endogenous signals that is implicated in eliciting induced direct defense against herbivores and pathogens (Wasternack and Parthier, 1997). In addition, exogenous application of JA induces the production of volatile compounds similar to HIPV (e.g., Hopke et al., 1994; Boland et al., 1995; Dicke et al., 1999; Gols et al., 1999; Ozawa et al., 2000), and HIPV emission is positively correlated to increased JA levels in corn plants during *Spodoptera exigua* herbivory (Schmelz et al., 2003).

Given the impact of JA on HIPV production, the obvious question is whether it can be used in the field to improve the effectiveness of natural enemies. Some encouraging first results have been obtained, showing that JA-treatment increases the attractiveness of otherwise clean plants to natural enemies of herbivorous arthropods. Detached Lima bean leaves and intact *Gerbera* plants became attractive to predators of spider mites under laboratory conditions (Dicke et al., 1999; Gols et al., 1999; Shimoda et al., 2002), and JA-treated tomato plants attracted parasitoids of beet armyworms in the field (Thaler, 1999). In the latter study, herbivores were present, but they were kept separate from the plant in Petri dishes with artificial diet. This was an elegant method to show that JA-treatment alone (i.e., irrespective of the impact of herbivory on the plant) increases the attractiveness of a plant to parasitoids and parasitization of the herbivore. However, for JA-treatment to be effective in promoting natural pest control, it is not only important to show that there are more natural enemies immigrating into the crop, but also to assess whether these enemies discriminate between herbivore-infested and uninfested plants within the crop. In theory, it is possible that JA-treatment causes natural enemies to waste searching time on JA-treated uninfested plants. Herbivores may be more difficult to find when their exact location on the plant and in the crop is not "marked" by HIPV, but becomes "diluted" in a cloud of HIPV emanating from the JA-treated crop as a whole. A positive effect of JA-treatment on parasitization rates will depend on the balance between increased immigration and reduced within-crop searching efficiency of natural enemies. For these reasons, we investigated (1) the comparative attractiveness of JA-treated, intact plants and JA-treated, infested plants by using untreated plants as a control, and (2) how differential attractiveness of the plant influences the herbivore's rate of mortality due to natural enemies. In addition, we assessed how long the effects of JA-treatment last (3). The tritrophic system under study consists of corn plants, the common armyworm *Mythimna separata* Walker, and its specialist parasitoid *Cotesia kariyai* Watanabe.

METHODS AND MATERIALS

Plants and Insects. In 2001, *Mythimna separata* was introduced to the laboratory from a culture reared at the National Institute of Sericultural and Entomological Science in Tsukuba, Ibaraki, Japan. The insects were reared on artificial diet (Insecta LF, Nihon Nousan Kogyo Ltd.) in the laboratory under conditions of 25 ± 2°C, 16L:8D, 50–70% RH.

Cotesia kariyai is a gregarious endoparasitoid of 2nd to early 6th instars of *M. separata* caterpillars. In 2001, the wasp was introduced to the laboratory from a stock culture reared at the Institute of Agriculture and Forestry, University of Tsukuba. To maintain the wasp culture, 3rd to 4th instars of *M. separata* are offered to female wasps for oviposition. Soon after emergence from their host, the wasp larvae spin a cocoon. Clusters of cocoons were placed in a glass tube ($\phi = 22$ mm, length = 200 mm). To provide a source of energy to the adult wasps emerging from the cocoons, a piece of cotton wool impregnated with a sugar solution was provided inside the glass tube. Mating occurred immediately after emergence. Adult wasps were stored in the laboratory under conditions of 18 ± 2°C, 50–70% RH, and continuous dark until used in the experiments within 7 d after reaching adulthood. Oviposition-inexperienced females were used to test innate responses.

Potted corn plants (*Zea mays* L. cv. Royal Dent) (three plants per pot) were grown in a greenhouse (25 ± 3°C, 16L:8D), and used for the experiments by the time they were 10 d old.

Flight Responses of Parasitoids to JA-Treated Corn Plants. The flight responses of female *C. kariyai* were observed with respect to two groups of three corn plants, each group in a different pot and receiving a different treatment. Two pots were positioned in a cage (25 × 35 × 30 cm) with three windows covered by nylon gauze and one door for introducing plants and wasps (Figure 1), and the cage was placed in a climate-controlled room (25 ± 2°C, 50–70% RH) (Shiojiri et al., 2000). Five to ten wasps at a time were released halfway between the two pots. Pots in the cage were replaced every 12–34 trials. The first landing by each wasp on a plant in either of the two pots was recorded as its choice. The wasp—once landed on a plant—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. Two replicates were performed, each with 12–34 wasps per test. Significant preferences in dual-choice tests were analyzed using a replicated *G*-test (Sokal and Rohlf, 1995; see page 715 and Box 17.4) 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 (Shiojiri, unpublished data), we found that releasing 5–10 wasps at the same time did not significantly affect the choice of the individual wasps.

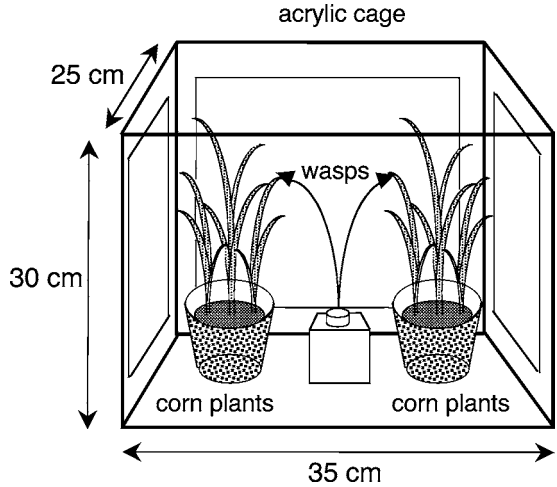

FIG. 1. Set-up of the experiment to assess the flight response of parasitic wasps to corn plants. Two pots, each with three plants and each receiving a different treatment, were positioned in a cage. Wasps were released halfway between two pots.

Three plants per pot, simultaneously received a combination of the following three treatments:

1. Spraying with 1.5 ml of an aqueous solution of JA (1 mM) or with 1.5 ml of distilled water (DW).
2. No herbivore infestation (hence no damage), or infestation by 10 third-stadium *M. separata* larvae for a period of 18 hr, after which the larvae and their feces were removed, leaving behind a damaged area of ca. 10% of the total leaf surface.
3. Timing of infestation at either day 1 or day 9 since spraying (see treatment 1), after which the plants were used for the bioassay.

Thus, plants were either labeled as DW-treated or JA-treated, and either as infested or uninfested. The duration between a treatment and a bioassay is given within brackets. For example in JA-treated, infested plants (10 d), larvae were released on the JA-treated plants 9 d after the treatment, and then 18 hr after their inoculation, the larvae and their associated products (such as feces) were carefully removed. Next, these plants were immediately used for the choice test.

Armyworm Parasitization Rate on JA-Treated Plants. We measured the rate at which armyworms were parasitized on JA-treated plants and DW-treated plants. Twenty third-stadium *M. separata* larvae were placed randomly on 12 plants in four pots (three per pot). Plants in two pots were JA-treated, and those in the

other two pots were DW-treated. Although there are cases where the exogenous application of JA to plants increases their defense against herbivores (e.g., Cipollini and Redman, 1999; Choh et al., 2004), we did not find any noticeable difference in the amount of damage by *M. separata* between JA-treated plants and DW-treated plants. After 18 hr, approximately 10% of the leaf surface of the plant had been consumed irrespective of whether the plant was treated with DW or JA. Two iron mesh cages (25 × 20 × 32 cm; ca. 1 mm mesh), one with two pots each with three JA-treated plants and another with two pots each with three DW-treated plants were placed, 1 m apart, in the center of the climate room (3.2 × 2.7 × 2.2 m; 25 ± 2°C; 60% RH; 2150 lx). As we confined the infested plants within the iron mesh cage, the wasps had to move through the 1 mm mesh gauze to attack the larvae. Average wind speed in the climate room was 0.1 m/sec (measured by a hot-wire anemometer). Twenty oviposition-inexperienced females were released into the space between the two cages at 10:30 AM. After 6 hr, the armyworm larvae were collected from the plants, and they were reared to the stage at which we assessed whether the caterpillar was parasitized or not.

When *C. kariyai* has succeeded in parasitizing a given host larva, it cannot immediately attack another larva. Successive parasitizations usually occur at intervals of 6–8 hr (Fukushima, unpublished data). Since the experimental period was 6 hr, such successful wasps would usually not parasitize again, but we cannot exclude that some wasps, especially those with early success, attacked another host in either of the two cages during the experimental period. Thus, the number of parasitizations may not represent fully independent trials. To circumvent this problem, we calculated the proportion of hosts parasitized on JA-treated plants out of all hosts parasitized on two groups of treated plants for each replicate experiment. If the number of hosts parasitized on JA-treated plants was higher than DW-treated plants, we scored a "+", and if lower, we scored a "–". Ties (i.e., the number of hosts parasitized on JA-treated plants were the same as that on DW-treated plants) were ignored for statistical analysis. These data were then subject a sign test against the null hypothesis that "+" and "–" are in a ratio of 1:1. We repeated the experiment seven times.

RESULTS

Flight Responses of Parasitoids to JA-Treated Corn Plants. We confirmed that infested plants were more attractive to *C. kariyai* than intact plants in the choice chamber (Figure 2, Table 1). When we compared the JA-treated intact plants (2 d) vs. DW-treated intact plants (2 d), significantly more wasps preferred the former plants (Figure 2, Table 1). When JA-treated infested plants (2 d) were compared to DW-treated infested plants (2 d), significantly more wasps again preferred the former (Figure 2, Table 1). Although the significance level was just

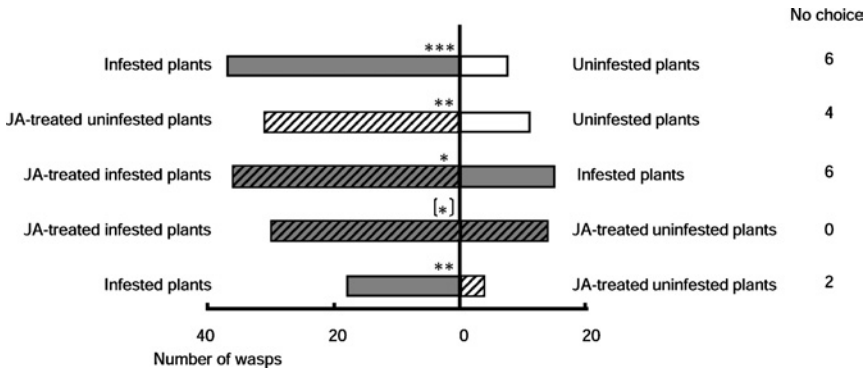

FIG. 2. Preference of *Cotesia kariyai* to fly to one of two alternatively treated corn plants in a small choice chamber (30 × 35 × 25 cm). Plants were subject to armyworm infestation or not (grey vs. white columns), and JA-treatment or not (columns with or without stripes) and any combination thereof. ***: $0.001 > P$; **: $0.001 < P < 0.01$; *: $0.01 < P < 0.05$ (replicated G-test, see Table 1).

on the edge ($P = 0.05$), we concluded that the wasps also preferred JA-treated infested plants (2 d) to JA-treated intact plants (2 d) (Figure 2, Table 1). Further, the wasps preferred DW-treated infested plants (2 d) over JA-treated intact plants (2 d) (Figure 2, Table 1).

JA-treated intact plants (10 d) were still more attractive than DW-treated intact plants (10 d) (Figure 3, Table 2). When JA-treated infested plants (10 d) were compared with DW-treated infested plants (10 d), significantly more wasps preferred the former plants (Figure 3, Table 2).

Armyworm Parasitization Rate on JA-Treated Plants. Six out of seven replicates were a "+", and the one remaining replicate resulted in a tie: proportion parasitized was exactly 0.5. Ignoring the tie ($N = 6$), the number of host caterpillars parasitized by wasps was significantly higher on JA-treated plants than on DW-treated plants according to a sign test (Figure 4; binomial test; $P = 0.016$). The actual rates of parasitism of host larvae on JA-treated infested plants and those on DW-treated infested plants are shown in Table 3.

DISCUSSION

The most striking result is that treating plants with JA does not interfere with the ability of parasitoids to discriminate olfactorily between caterpillar-infested plants and uninfested plants. Two days after plant treatment, the rank order of attractiveness to parasitoids increased from DW-treated intact plants, JA-treated intact plants, DW-treated infested plants to JA-treated infested plants (Figure 1).

TABLE 1. SUMMARY OF THE REPLICATED *G*-TESTS FOR PREFERENCE OF *Cotesia kariyai* TO FLY TO ONE OF TWO ALTERNATIVELY TREATED CORN PLANTS

Treatment	*G* statistics		
	Pooled[a]	Heterogeneity[b]	Total[c]
Infested plants vs. Uninfested plants	20.26***	0.51	20.77***
JA-treated uninfested plants vs. DW-treated uninfested plants	9.92**	1.12	11.04**
JA-treated infested plants vs. DW-treated infested plants	8.91**	0.05	8.96*
JA-treated infested plants vs. JA-treated uninfested plants	5.95*	0.03	5.99(*)
DW-treated infested plants vs. JA-treated uninfested plants	9.64**	0.86	10.50**

Note. The data from which this table is prepared are shown in Figure 1. ***: $P < 0.001$; **: $0.001 < P < 0.01$; *: $0.01 < P < 0.05$; (*): bordering significant ($P = 0.05$).
[a] df = 1.
[b] df = 1.
[c] df = 2.

The effect of JA-treatment was still present at least 10 d after the treatment, which reconfirms earlier results by Thaler (1999). Gols et al. (2003) have reported recently that volatiles emitted from JA-treated Lima bean plants subsequently infested with spider mites attracted natural enemies of the herbivore more than those emitted from spider mite-infested plants, and the effects of JA lasted at least 7 d. These results support our results with corn plants, caterpillars, and wasps. In addition, our study complements the olfactometer study of Gols et al.

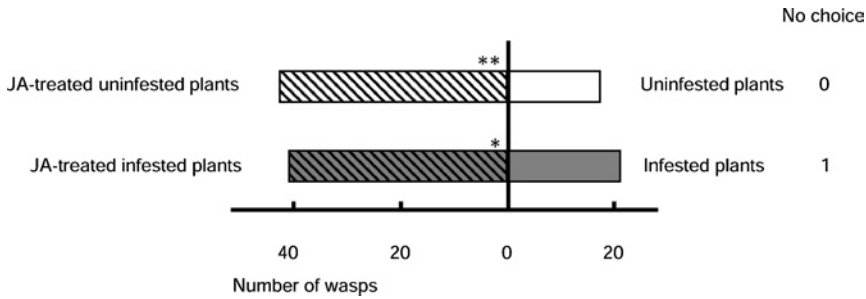

FIG. 3. Preference of *Cotesia kariyai* to fly to one of two alternatively treated corn plants (as in Figure 2), but now assessed 10, instead of 2, days after treatment. Asterisks refer to significance levels of *G*-tests, as in Figure 2 (for replicated *G*-test, see Table 2).

TABLE 2. SUMMARY OF THE REPLICATED *G*-TESTS FOR PREFERENCE OF *Cotesia kariyai* TO FLY TO ONE OF TWO ALTERNATIVELY TREATED CORN PLANTS

Treatment	*G* statistics		
	Pooled[a]	Heterogeneity[b]	Total[c]
JA-treated uninfested plants vs. DW-treated uninfested plants	11.56**	0.26	11.82**
JA-treated infested plants vs. DW-treated infested plants	6.57*	0.07	6.64*

Note. The data from which this table is prepared are shown in Figure 2. Asterisks refer to significance levels of replicated *G*-tests, as in Table 1.
[a] df = 1.
[b] df = 1.
[c] df = 2.

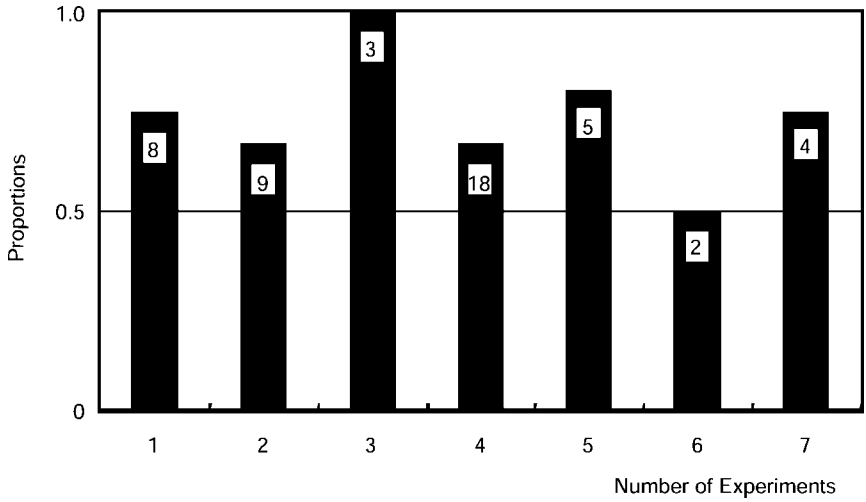

FIG. 4. Proportions of *Mithimna separata* larvae parasitized by *Cotesia kariyai* on JA-treated infested corn plants out of all host larvae parasitized on JA-treated infested corn plants and on DW-treated infested corn plants in a climate room. The number of all hosts parasitized in two groups of plants in individual experiments is given at the top of each bar. The proportions of parasitization obtained from the 6 replicates were evaluated statistically, using a binomial sign test. Since in the 6th replicate the number of hosts parasitized on JA-treated plants was exactly the same as that on DW-treated plants, this replicate was not included in the statistical analysis.

TABLE 3. RATE OF PARASITISM BY *Cotesia kariyai* OF *Mithimna separata* LARVAE ON JA-TREATED INFESTED CORN PLANTS AND ON DW-TREATED INFESTED CORN PLANTS

	Treatment	Number of released larvae (A)	Number of parasitized larvae (B)	Parasitic rate [$A/B \times 100$ (%)]
1	JA	20	6	30
	DW	20	2	10
2	JA	20	6	30
	DW	20	3	15
3	JA	20	3	15
	DW	20	0	0
4	JA	20	12	60
	DW	20	6	30
5	JA	20	4	20
	DW	20	1	5
6	JA	20	1	5
	DW	20	1	5
7	JA	20	3	15
	DW	20	1	5

(2003), by investigating whether pretreatment of plants with JA influences the herbivore's rate of mortality on a spatial scale where parasitoids have to locate their hosts from a distance, i.e., a climate room of 3.2 × 2.7 × 2.2 m. The results of these parasitization experiments demonstrate that the increased attractiveness of JA-treated infested plants observed in a small choice chamber (30 × 35 × 25 cm) translates into increased armyworm parasitization in a larger space (i.e., a climate room). This suggests that uniform application of JA to agricultural crops causes the crop to be more attractive to parasitoids in the environment, and that, once in the crop, the attracted parasitoids aggregate on individual plants that are caterpillar-infested. This hypothesis needs to be tested in the field, but our experiments show that at least some of the conditions are fulfilled for JA-treatment of crops to be effective in practice: i.e., JA-treatment of plants does not interfere with *long-distance* host location and has a lasting effect on a treated plant.

However, there are many other conditions left unexplored, and these require tests in the laboratory and/or large-scale tests in the field. For example, JA-treatment may interfere with *short-distance* host location within a plant, if the exact location of the host is hidden in the odor plume emanating from the JA-treated, caterpillar-infested plant. This is an important question that also has a bearing on the impact of systemic release of HIPV by partially infested plants. Also, our lack of understanding of the mechanisms that cause increased attractiveness of JA-treated plants needs exploring. We will report on the proximate

factors involved in increased attractiveness (i.e., odor blend composition) in a forthcoming paper.

Finally, more research is needed to assess the costs of JA-induced defense apart from the benefits identified in this article (Baldwin, 1998; Thaler, 1999; Redman et al., 2001). For a proper evaluation of treating crops with JA, a complete cost–benefit analysis is mandatory.

That JA-treatment of infested plants promotes a plant's attractiveness, is also striking from another perspective. The corn plants used in our study underwent artificial selection by breeding in the recent past and natural selection in their ancestral past. How the plant breeding process affected the plant traits relevant for attracting parasitoids is not immediately obvious and requires research (Lindig et al., 1997). One may hypothesize that crop plants represent better food sources for herbivorous arthropods and thereby also better prey/hosts for predators/parasitoids. Consequently, natural selection acting on the herbivore's enemies may have promoted the ability to locate prey/hosts on crop plants (Benrey et al., 1998; González-Rodríguez et al., 2000). On the other hand, plant breeding is a human activity, which has so far not been guided by the idea of plants attracting bodyguards. How the process of plant domestication has influenced induced attractiveness of plants to parasitoids is not clear. One might interpret the improved attractiveness of plants after JA-treatment as an indication that there is still room for ameliorating crops! This interpretation is in agreement with Loughrin et al. (1995) who found lower production of HIPV in cultivated cotton cultivars than in a naturalized cotton cultivar. Moreover, it is in line with our observation that JA-treated infested corn plants are more attractive than DW-treated infested plants. However, there are alternative explanations for the increased attractiveness of JA-treated infested plants. For example, if JA-levels in plants are not fully under control of the infested plant, but down-regulated by specific actions of the herbivore, then external application of JA may reduce the control of the herbivore over the JA-levels in the plant, and this may make the plant more attractive. Future research should reveal which of these alternative explanations stands up to scrutiny.

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FIELD CAPTURE OF NORTHERN AND WESTERN CORN ROOTWORM BEETLES RELATIVE TO ATTRACTANT STRUCTURE AND VOLATILITY

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Abstract—We used field assays to study attraction of feral northern and western corn rootworm beetles (*Diabrotica barberi* and *D. virgifera virgifera*) to a series of mostly nitrogenous and benzenoid synthetic compounds allied with host plant and floral aromas. Vaporization rates were obtained for most field-tested compounds and selected additional lures under both ideal and field-representative, but constant, conditions. Although many test compounds showed at least trace activity for one or both species, methyl benzoate and some of its derivatives, notably methyl anthranilate and methyl 4-methoxybenzoate, merited emphasis as effective new lures for females. Structural alteration of methyl benzoate had consistently negative effects on northern corn rootworm captures despite variable effects on release rate, whereas western corn rootworm was more strongly attracted to methyl anthranilate and methyl 4-methoxybenzoate than to the considerably more volatile parent compound. Phenylacetaldoxime was attractive to females of both species, but no more so than *syn*-benzaldoxime, included as reference. Release rate was disproportionately low for benzaldoxime, as well as other nitrogenous lures, under field compared with ideal conditions. The attractiveness of salicylaldoxime to northern corn rootworm, despite its low field release rate, and the unattractiveness of methyl salicylate, having a methyl ester in place of the oxime group, similarly highlighted importance of the oxime moiety for reactivity of this species.

Key Words—*Diabrotica barberi*, *Diabrotica virgifera virgifera*, corn rootworm, kairomone, attractant, lure volatility, trapping, phenylacetaldoxime, methyl benzoate, methyl anthranilate, methyl 4-methoxybenzoate.

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INTRODUCTION

Diabroticite corn rootworms are key pests of maize, *Zea mays* L. Crop injury in the Midwest corn belt of the United States derives mostly from larval root feeding by northern and western corn rootworm, *Diabrotica barberi* (Smith and Lawrence) and *D. virgifera virgifera* (LeConte), respectively. Western corn rootworm was also introduced into Europe in the 1990s, where its spread poses a growing threat to maize production (Enserink, 1999). Both species prefer to feed as adults on pollen, silks, and young kernels of maize (Branson and Krysan, 1981), providing the potential for additional crop damage at high pest density. Western corn rootworm utilizes maize foliage when the preferred foods become unavailable, whereas northern corn rootworm visits a variety of flowering forbs seeking pollen (Branson and Krysan, 1981; Metcalf and Lampman, 1997). Adults also exploit blossoms of cucurbits, descendents of hypothetical ancestral diabroticite hosts, when available (Metcalf and Metcalf, 1992). Host plant volatiles from both maize and cucurbit blossoms, a number of their analogs, and some floral odorants attract adults in species characteristic patterns (Lance, 1993; Metcalf and Lampman, 1997; Hammack, 2003; references in each citation). These compounds have potential applications in pest population monitoring and control via such tactics as mass annihilation of reproductive adults, especially given the strong bias toward female captures.

A previous study showed that *syn*-benzaldoxime attracted both northern and western corn rootworm (Hammack, 2001). The oxime attracted about twice as many *D. barberi* of both sexes as did cinnamyl alcohol, a potent northern corn rootworm lure of cucurbit blossom origin (Metcalf and Lampman, 1989a). This oxime was initially tested because of its structural similarity to two benzenoid lures: 2-phenyl-1-ethanol, released by maize and cucurbit blossoms and moderately attractive to both rootworm species, and 2-phenyl-1-ethylamine, an effective northern corn rootworm attractant (Metcalf and Lampman, 1991) not known from host plants but apparently acting synergistically with the alcohol and active enough to alter beetle distributions within maize fields before most females would have oviposited (Petroski and Hammack, 1998; Hammack, 2003). Neither the oxime nor the amine is reported from host plants of diabroticites, but a related compound, phenylacetaldoxime, is common in floral aromas (Kaiser, 1991; Knudsen et al., 1993).

Volatility estimates obtained by measuring release rates from thin films on aluminum planchets are available for some diabroticite lures of squash-blossom origin or their structural analogs (Metcalf and Lampman, 1991; Metcalf and Metcalf, 1992). Most studies of kairomones affecting *Diabrotica* adult behaviors, however, dispense test compounds from cotton wicks, and lure release rates are generally unavailable for cotton dispensers despite their widespread

use (Metcalf and Metcalf, 1992; Lance, 1993; Metcalf et al., 1995; Ventura et al., 2000; Hammack, 2003; references in each citation).

Here, we evaluated attraction of feral northern and western corn rootworm beetles to benzaldoxime compared with phenylacetaldoxime and to a series of typically nitrogenous and/or benzenoid synthetic compounds structurally related to the oximes or to methyl benzoate. Volatility and release rate data were obtained for most of the candidate attractants, as well as for several compounds previously shown to be attractive when vaporized from cotton-wick dispensers, to facilitate evaluation.

METHODS AND MATERIALS

Candidate Attractants. Source and purity of test compounds are listed in Table 1, except for phenylacetaldoxime. The oxime was synthesized from phenylacetaldehyde and hydroxylamine hydrochloride, as per Vogel (Furniss et al., 1989). Purity was determined to be >95% (GC analysis) after recrystallization (3×) from benzene–hexane.

Volatility and Release Rate Determinations. Test compound volatility was determined gravimetrically using the rate of weight loss from a 20-mg loading dose in 100–200-μl acetone dispersed over aluminum planchets exposed in a laboratory hood at a wind speed of about 45 m min^{-1}, as detailed by Metcalf and Lampman (1991). Nominal release rate from cotton-wick dispensers was determined under the same conditions but at higher loading dose (Table 1) or over a dosage range (Table 2), and only solids were applied in solvent, as described below for the field experiments. Release from cotton dispensers was also measured at 20–23°C, 2–3°C cooler than that from aluminum. Cotton wicks were obtained from Patterson Dental Supply Co. (Minneapolis, MN, No. 085-0073 cotton rolls, 1-cm diam. × 3.8-cm long). Controls for possible weight change of the absorbent dispensers due to changes in relative humidity during testing were also included, as previously described (Hammack, 2003). Weight loss determinations were replicated four times for each dispenser type and dosage. Slopes of lines relating mean weight of volatile-treated dispensers with time after treatment were used to estimate compound volatility and nominal release rates.

Bioassays and Experiments. Attractiveness of test chemicals to northern and western corn rootworm beetles was assessed using beetle captures on baited and unbaited yellow sticky traps (Pherocon AM, Trécé Inc., Salinas, CA) wrapped around maize plants at about ear height in commercial maize fields in Brookings County, SD. Compounds solid below 35°C were dissolved in 100–200-μl ethanol, or acetone in the case of indole, before application to the cotton dispensers used in all field trials. All dispensers on traps within tests received the same solvent

TABLE 1. TEST CHEMICAL SOURCE, PURITY AND RELEASE RATE BY DISPENSER LOADING DOSE AND TYPE

Chemical	Source/purity (%)	Release rate (μmoles hr^{-1})		
		20 mg on aluminum	100 mg on cotton	600 μmoles on cotton
Methyl benzoate	Aldrich/99	286.45	133.44	108.29
Phenyl acetate	Aldrich/99	175.78	—	56.50
Methyl 2-methylbenzoate	Aldrich/99	125.09	58.33	—
Ethyl benzoate	Aldrich/>99	126.99	—	54.54
Benzyl acetate	Aldrich/>99	74.34	—	37.92
Methyl 4-methylbenzoate	Aldrich/>99	119.19	—	34.83
Propyl benzoate	Aldrich/99	37.47	31.47[a]	31.47
Methyl salicylate	Aldrich/>99	168.26	33.28	28.35
Methyl phenylacetate	Aldrich/>99	78.65	—	25.64
Ethyl phenylacetate	Aldrich/>99	40.91	21.58[a]	21.58
2-Phenethyl acetate	Fluka/≥99	30.00	16.66[a]	16.66
2-Phenyl-1-ethanol	Aldrich/99	37.37	13.30	—
β-Caryophyllene	Fluka/99	20.44	11.55	—
Geranylacetone	Fluka/>98	8.40	4.04	—
Indole	Aldrich/>99	39.33	3.46	—
Methyl anthranilate	Aldrich/>99	13.37	2.53	2.59
syn-Benzaldoxime	Fluka/99	24.27	2.17	—
Ethyl *trans*-cinnamate	Aldrich/99	6.08	2.10	2.41
Methyl 2-methoxybenzoate	Aldrich/99	5.74	1.94[a]	1.94
Methyl 4-methoxyphenylacetate	Aldrich/97	2.89	—	1.03
Methyl 4-methoxybenzoate	Aldrich/99	9.24	0.89[a]	0.89
Salicylaldoxime	Aldrich/98	2.44	0.43	—
Cinnamyl alcohol	Aldrich/98	1.93	0.94	—
4-Methoxycinnamaldehyde	Schw[b]/≥98	0.15	0.07	—
(±)-Linalool	Aldrich/97	184.11	—	—
2-Phenyl-1-ethylamine	Aldrich/>99	102.93	—	—
(+)-α-Terpineol	Fluka/99	55.94	—	—
β-Ionone	Aldrich/96	0.16	—	—
Methyl 4-aminobenzoate	Aldrich/98	0.04	—	—
Methyl 2-nitrobenzoate	Aldrich/98	—	—	—
Methyl 4-methoxysalicylate	Aldrich/98	—	—	—
2,1-Benzisoxazole	Aldrich/99	—	—	—
1,2-Benzisoxazole	Aldrich/95	—	—	—
Pyrrole	Aldrich/98	—	—	—
Acetone	Aldrich/>99.5	—	—	—
Hexane	Aldrich/>99	—	—	—

[a]Estimated from the 600 μmole treatment weighing 97.5 ± 2.2 mg.
[b]Schweizerhall, Inc. South Plainfield, NJ.

TABLE 2. RELATIVE RELEASE RATE FOR SELECTED ATTRACTANTS AS A FUNCTION OF DOSE APPLIED TO COTTON DISPENSERS (RATE IN μmoles hr^{-1})

Attractant	Dose (mg dispenser^{-1})		
	10	30	100
Methyl benzoate	0.24	0.40	1 (133.44)
Methyl salicylate	0.31	0.56	1 (33.28)
2-Phenyl-1-ethanol	—	0.44	1 (13.30)
β-Caryophyllene	0.36	0.64	1 (11.55)
Methyl anthranilate	0.41	0.64	1 (2.53)
syn-Benzaldoxime	0.28	0.52	1 (2.17)
Ethyl *trans*-cinnamate	0.29	0.49	1 (2.10)
Salicylaldoxime	0.67	0.86	1 (0.43)

quantities, but none of the extender that was previously employed (Hammack, 1996, 2001). Traps were separated from each other and field edges by 30 m or more. Maize phenology and beetle number per plant were estimated on the last day of each test from 40 plants spread throughout test areas but located about midway between trap sites. Traps were returned to the laboratory where captured corn rootworm beetles were counted by species and sex. Chemicals were deemed olfactorily attractive if they caught significantly more beetles than did a control.

Each of three field experiments was laid out in a randomized complete block design with six replicates. A 1997 test compared beetle responses to *syn*-benzaldoxime and phenylacetaldoxime at dispenser loading doses from 1 to 300 mg. A 1998 test examined responses to methyl benzoate, to a series of its derivatives, and to nitrogenous compounds structurally related to this ester or to the oximes. The 1997 and 1998 tests each lasted 48 hr. A shorter duration test deployed for 24 hr in 2000 further examined effects of functional group additions to the methyl benzoate ring and modification of its side chain. Dispenser loading dose was 100 mg in the 1998 test but changed to 600 μmoles in 2000. An equimolar loading dose would theoretically ensure an olfactory stimulus of quantitatively equivalent strength for compounds of similar volatility but different molecular weight.

Statistical Analysis. Data analyses used SAS statistical software (SAS Institute, 1989). Linear regression analysis calculated the slope of lines describing rate of loss of test compounds from dispensers. For each species and sex within field tests, trap capture data were transformed [$\ln(x + 1)$] to meet the assumption of variance homogeneity before using analysis of variance (ANOVA) to test the null hypothesis of no difference in captures among lure treatments. A Student–Newman–Keuls test separated means after a significant ANOVA ($P \leq 0.05$). Figures show untransformed data.

RESULTS

Volatility and nominal release rate data obtained for most attractants examined here and for a few lures tested previously, including cinnamyl alcohol, geranylacetone, and linalool (Hammack 1996, 2001), showed that release rates from cotton, while slower, generally tended to correlate with those from aluminum planchets, as expected (Table 1). However, there were notable exceptions. Most striking, a switch from aluminum to cotton reduced rates disproportionately for all of the nitrogenous compounds (benzaldoxime, salicylaldoxime, indole, and methyl anthranilate) and for methyl 4-methoxybenzoate and methyl salicylate. A similar albeit weaker reduction occurred for phenyl acetate, methyl phenylacetate, methyl 4-methoxyphenylacetate, methyl 2-methoxybenzoate, methyl 4-methylbenzoate, 2-phenyl-1-ethanol, and ethyl *trans*-cinnamate (Table 1). As a result, the release rate of benzaldoxime from cotton was only about twice that of cinnamyl alcohol, despite the more than 10-fold difference in volatility when vaporization was from thin films on aluminum planchets.

For the attractants ordered in Table 2 from most to least volatile, release rates following application of 10, 30, and 100 mg to cotton varied to a lesser degree than did the dispenser loading doses. With the exception of salicylaldoxime, the least volatile of the test lures, the 10-fold range in loading dose resulted in a 2.4- to 4.2-fold change in release rate. Salicylaldoxime release rate varied by only 1.5 times under the same conditions. Otherwise, there was little relationship between compound volatility and the variation in release rate with change in loading dose (Table 2).

Loss of weight of cotton wicks with time after methyl anthranilate treatment (Figure 1) illustrates several key points about the determination of release rates shown in Tables 1 and 2. First, release of test volatiles occurred in the laboratory at constant linear rates that continued until wicks lost more than 70–80% of the loading dose (e.g., Figure 1, 10-mg dose) or until observation was ended after more than 48 hr, the longest field-trapping interval used here. In addition, regression analyses that generated the cotton-wick data in Tables 1 and 2 were always highly significant ($P < 0.001$) and produced r^2 values usually ≥ 0.98. Four lower r^2 values occurred: 0.91 for 4-methoxycinnamaldehyde, which was observed for only 5 d despite its very low volatility, and 0.95–0.97 for 10-mg doses of methyl benzoate, ethyl *trans*-cinnamate, and methyl salicylate.

Northern and western corn rootworm females were attracted by both benzaldoxime and phenylacetaldoxime, although only the higher phenylacetaldoxime doses attracted the latter (Figure 2). No data are shown for males because their numbers in no case differed significantly between oxime and control treatments. For females, mean captures varied significantly with dose of benzaldoxime, but only western corn rootworm showed a dose-dependent response to phenylacetaldoxime. Respective regression equations for each lure ($N = 5$), where

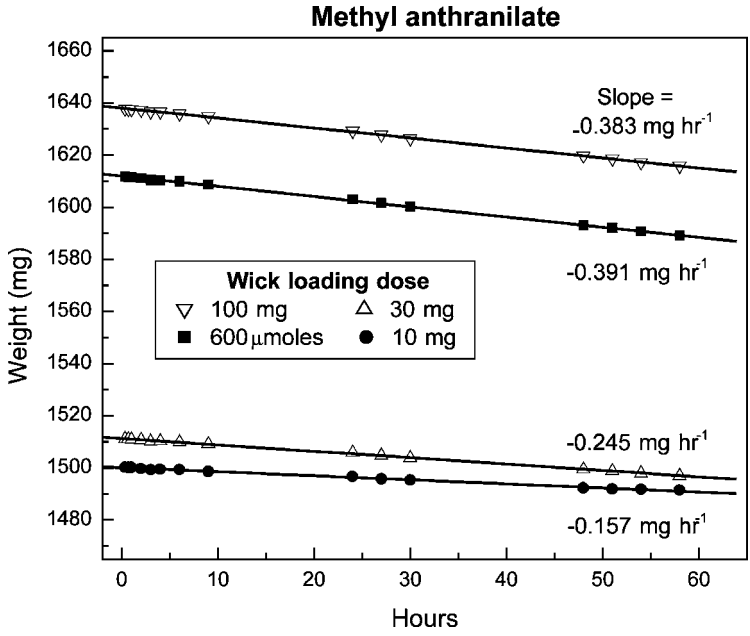

FIG. 1. Representative regression lines calculated to estimate release rates for different loading doses of compounds applied to cotton dental wicks.

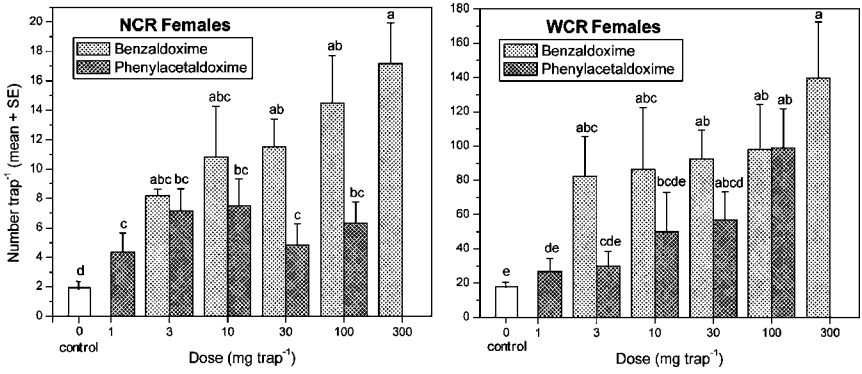

FIG. 2. Capture of female northern (NCR) and western corn rootworm (WCR) beetles after 48 hr on traps baited with benzaldoxime or phenylacetaldoxime at varied doses. Values topped by different letters differ at $P \leq 0.05$ by Student–Newman–Keuls test after ANOVA ($F_{NCR} = 9.40$, $F_{WCR} = 7.46$; df = 10, 50; and $P < 0.001$ for both species). At test end, August 28, 1997, maize was dough stage (R4) and mean beetle count per plant (±SE) was 0.7 ± 0.2 and 1.1 ± 0.2 for NCR and WCR, respectively ($N = 40$).

$x = \ln(\text{dose})$ and $y = \ln(\text{mean capture trap}^{-1} + 1)$, were $y = 0.13x + 2.06$, $r^2 = 0.99$, $P < 0.001$ (northern); $y = 0.14x + 3.96$, $r^2 = 0.77$, $P = 0.05$ (western); and $y = 0.03x + 1.76$, $r^2 = 0.09$, $P > 0.6$ (northern); $y = 0.30x + 2.97$, $r^2 = 0.95$, $P < 0.005$ (western). The increasing responses of both species to the 10-, 30-, and 100-mg doses of benzaldoxime did not differ statistically (Figure 2), despite the 4-fold increase in nominal release rate achieved with the 10-fold increase in loading dose (Table 2).

Captures on traps exposed in 1998 and baited with a nitrogenous compound or with methyl benzoate or one of its derivatives showed three compounds to be especially effective lures for northern corn rootworm females: methyl benzoate (nominal release rate of 133.4 μmoles hr^{-1}), benzaldoxime (2.2 μmoles hr^{-1}) included as a reference, and salicylaldoxime (0.4 μmoles hr^{-1}) (Figure 3). Only methyl benzoate was significantly attractive to northern corn rootworm males. Compounds included in this test but omitted from Figure 3 because they failed to capture significantly more beetles of either sex or species than did control traps were methyl 2-nitrobenzoate, methyl 4-methoxysalicylate, 2,1-benzisoxazole (anthranil), 1,2-benzisoxazole, and pyrrole. Of the three best lures, methyl benzoate attracted the most and salicylaldoxime the fewest northern corn rootworm females; however, nominal release rates, which differed markedly between methyl benzoate and the oximes, declined in the same order and methyl benzoate likely dissipated well before the end of the 48-hr test period. Functional group addition in either the 2- or 4-position of the methyl benzoate ring dramatically reduced northern corn rootworm female captures despite a highly variable effect on release rate. Rates in micromoles per hour after functional group addition were 58.3 for methyl, 33.3 for hydroxyl, 2.5 for amino, and 1.9 for methoxy groups in the 2-position and 0.9 for a methoxy group in the 4-position. Methyl anthranilate (2-amino addition) and methyl 4-methoxybenzoate were the only test compounds to retain any attractiveness to northern corn rootworm females after functional group addition to the methyl benzoate ring (Figure 3). Cotton wicks treated with methyl anthranilate tended to develop a brownish-purple discoloration during this and subsequent field trials, although not in the laboratory tests.

All of the test compounds in Figure 3, except salicylaldoxime with a release rate of 0.4 μmoles hr^{-1}, captured significantly more western corn rootworm females than did control traps, but highest captures occurred in response to methyl 4-methoxybenzoate, followed by methyl anthranilate, benzaldoxime, indole, and methyl benzoate. The only compound attractive to western corn rootworm males was methyl 4-methoxybenzoate. Replacement of its 4-methoxy with a 2-methoxy or 2-amino group decreased captures, but significantly so only for females, despite small increases in release rates (Figure 3). Addition of a 2-methyl or 2-hydoxyl group to the methyl benzoate ring also depressed captures, although less than it did for northern corn rootworm and the reductions were not statistically significant for either western corn rootworm sex (Figure 3).

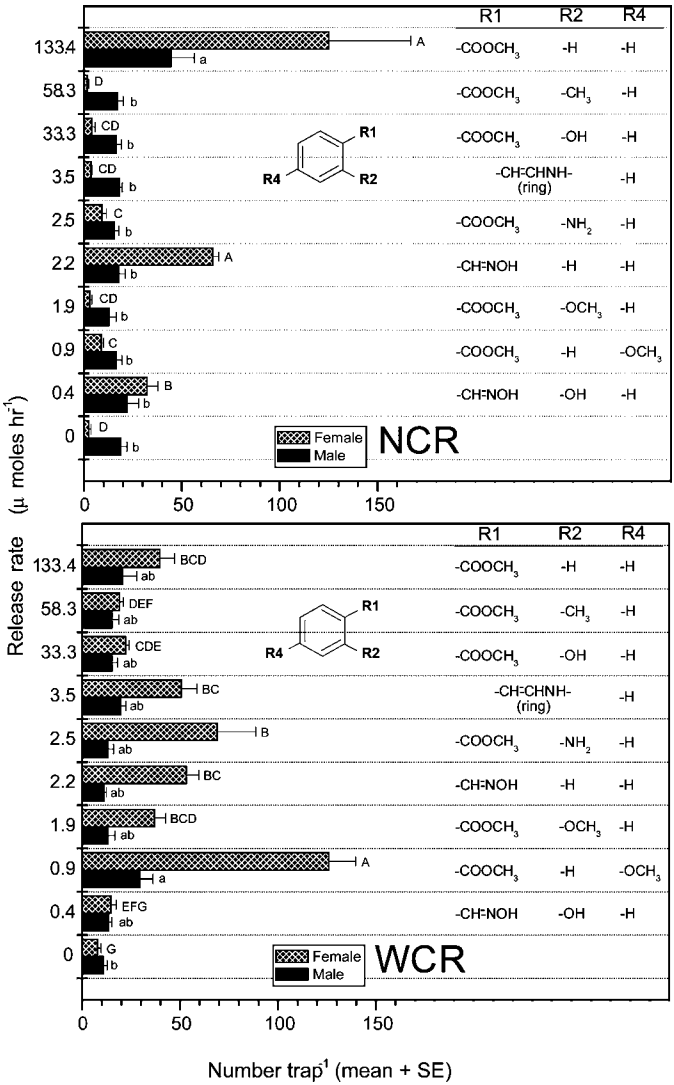

FIG. 3. Capture of northern (NCR) and western corn rootworm (WCR) beetles by sex after 48 hr in relation to lure structure and nominal release rate achieved with 100 mg on cotton dispensers. Rate 0 denotes control. Values within species and sexes topped by different letters differ at $P \leq 0.05$ by Student–Newman–Keuls test after ANOVA ($F_{NCR} = 24.76$ and $F_{WCR} = 17.84$ for females, $P < 0.001$; $F_{NCR} = 2.48$ and $F_{WCR} = 2.04$ for males, $P < 0.03$; df = 14, 70 for both sexes). At test end, August 27, 1998, maize was dent stage (R5) and mean beetle count per plant (±SE) was 1.7 ± 0.2 and 1.5 ± 0.2 for NCR and WCR, respectively ($N = 40$).

Functional group additions to the methyl benzoate ring or modification of its side chain generally reduced northern corn rootworm captures in 2000, when test duration was 24 hr instead of 48 hr (Figure 4). Methyl or amino addition in the 4-position obliterated attractiveness although the latter change also profoundly reduced release rate. The 2-amino and 4-methoxy additions led to lures still slightly attractive to northern corn rootworm females and, in contrast with 1998, capturing males in numbers just significantly above control. Ethyl and propyl esters of benzoic acid likewise captured significantly fewer beetles of both sexes than did the methyl ester; side-chain elongation by one carbon reduced release rates by $\leq$2-fold, probably too little to explain the lower captures (Figure 4). A striking fall in northern corn rootworm captures occurred with the methyl ester of phenyl acetic acid compared with that of benzoic acid, although release rate also fell about 4-fold (25.6 compared with 108.3 μmoles hr^{-1}). Still, it is unlikely that reduced efficacy arose entirely from the release rate drop because of its relatively small magnitude and because the 4-methoxy derivatives of these same esters also differed in attractiveness, but not in release rates (1.0 and 0.9 μmoles hr^{-1}). Phenyl acetate also captured many fewer northern corn rootworms of both sexes than did methyl benzoate, its structural isomer, with a nominal release rate about half that of the benzenoid ester (56.5 *vs.* 108.3 μmoles hr^{-1}). Despite little if any attractiveness of the acetate esters of phenol (56.5 μmoles hr^{-1}) and benzyl alcohol (37.9 μmoles hr $^{-1}$), the acetate ester of 2-phenyl-1-ethanol (16.7 μmoles hr^{-1}) was modestly attractive to northern corn rootworm females, although less so than was ethyl *trans*-cinnamate (2.4 μmoles hr^{-1}) (Figure 4). Ethyl *trans*-cinnamate captured about as many northern corn rootworms as did ethyl benzoate (54.5 μmoles hr^{-1}), but the latter much more volatile compound likely dissipated within 12 hr complicating comparison of attractant activities.

All of the compounds in Figure 4 caught significantly more western corn rootworm females than did the control, except for methyl 4-aminobenzoate with its low nominal release rate (<0.04 μmoles hr^{-1}). Highest captures were recorded in response to methyl anthranilate (2.6 μmoles hr^{-1}) and methyl benzoate (108.3 μmoles hr^{-1}), followed by methyl 4-methoxybenzoate (0.9 μmoles hr^{-1}), ethyl benzoate (54.5 μmoles hr^{-1}), ethyl *trans*-cinnamate (2.4 μmoles hr^{-1}), and 2-phenethyl acetate (16.7 μmoles hr^{-1}). Males responded only to methyl 4-methoxybenzoate, methyl anthranilate, and ethyl *trans*-cinnamate in numbers significantly greater than control, although many fewer males than females were captured. Western corn rootworm female captures declined with ethyl and propyl compared with the methyl ester of benzoic acid, in similarity with the northern corn rootworm response pattern (Figure 4). Also like northern corn rootworm patterns were the western corn rootworm decline in captures with phenyl acetate (56.5 μmoles hr^{-1}) compared with methyl benzoate (108.3 μmoles hr^{-1}) and the improved western corn rootworm captures with 2-phenethyl acetate (16.7 μmoles hr^{-1}) compared with phenyl or benzyl acetates (37.9 μmoles hr^{-1}).

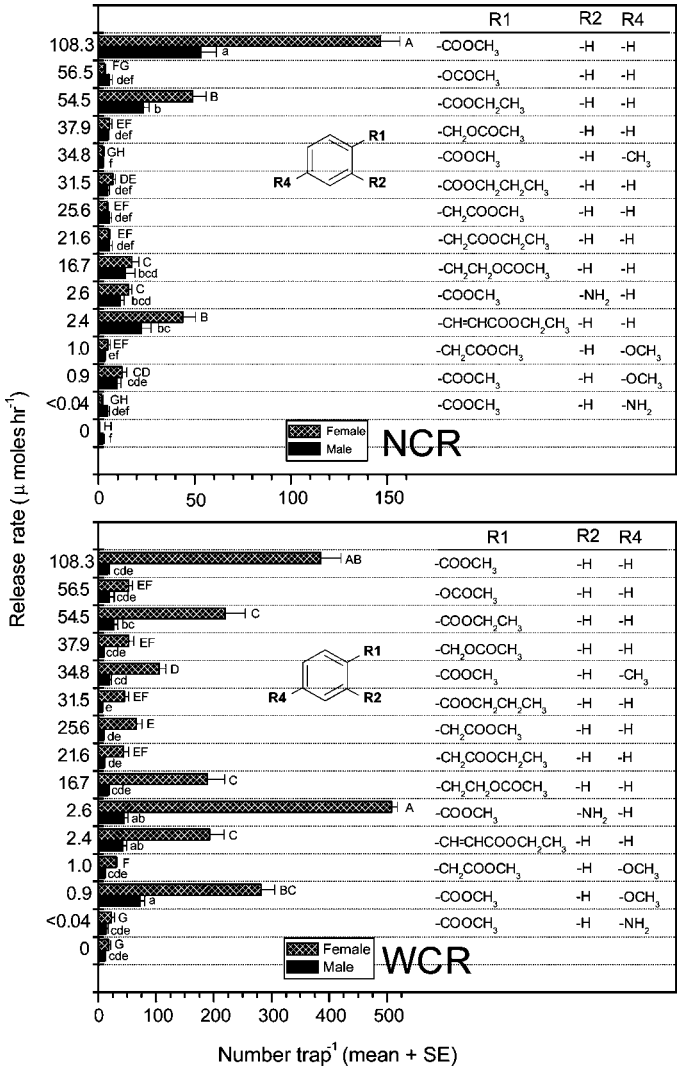

FIG. 4. Capture of northern (NCR) and western corn rootworm (WCR) beetles by sex after 24 hr in relation to lure structure and nominal release rate achieved with 600 μmoles on cotton dispensers. Rate 0 denotes control. Values within species and sexes topped by different letters differ at $P \leq 0.05$ by Student–Newman–Keuls test after ANOVA ($F_{NCR} = 50.99$ and $F_{WCR} = 61.26$ for females; $F_{NCR} = 14.68$ and $F_{WCR} = 12.02$ for males; $P < 0.001$ and df = 14, 70 for both sexes). At test end, August 31, 2000, maize was dough to dent stage (R4–R5) and mean beetle count per plant (±SE) was 1.8 ± 0.3 and 3.4 ± 0.5 for NCR and WCR, respectively ($N = 40$).

DISCUSSION

Our finding that benzaldoxime release rate from cotton was only about twice that of cinnamyl alcohol, deemed a potent northern corn rootworm lure (Metcalf and Metcalf, 1992), indicates that greater efficacy of the oxime than alcohol in past trials (Hammack, 2001) was not simply due to a higher oxime release rate. Female northern corn rootworm attraction to salicylaldoxime, despite its low nominal release rate (0.4 μmoles hr^{-1}), and insensitivity to the more volatile methyl salicylate (33.3 μmoles hr^{-1}) having an ester linkage in place of the oxime group, also accentuated oxime importance. Our results gave no hint that phenylacetaldoxime, at least as a mixture of stereoisomers, is any more attractive to northern or western corn rootworm beetles than is *syn*-benzaldoxime, despite the presence of the former in floral head-space volatiles (Kaiser, 1991; Knudsen et al., 1993). Fewer beetles of both species usually responded to the former, longer-chain, oxime, perhaps because of a lower release rate. We did not measure phenylacetaldoxime emission, but did show that similar one-carbon elongation of the side chain of related esters reduced nominal release rate by about 1.5- to 4-fold. This pattern manifested here for methyl benzoate (108.3 μmoles hr^{-1}) *vs.* methyl phenylacetate (25.6 μmoles hr^{-1}), phenyl acetate (56.5 μmoles hr^{-1}) *vs.* benzyl acetate (37.9 μmoles hr^{-1}), and benzyl acetate *vs.* 2-phenethyl acetate (16.7 μmoles hr^{-1}).

Our study showed that methyl benzoate and some of its structural analogs were attractive to both northern and western corn rootworm beetles, confirming earlier reports that methyl salicylate, with its 2-hydroxyl addition to the methyl benzoate ring, attracts western if not northern corn rootworm (Hammack, 2001). The methyl benzoate structure is apparently crucial for northern corn rootworm responses because various ring substitutions, side chain alterations, and a modified ester linkage all had consistently negative effects on lure efficacy despite variable effects on nominal release rates. Whereas the side chain and ester linkage changes were largely detrimental in both species, some additions at the 2- or 4-position of the methyl benzoate ring tended to maintain or improve western corn rootworm attraction. Methyl anthranilate, with its 2-amino addition, and methyl 4-methoxybenzoate, for example, were particularly effective lures given their much lower release rates than that of methyl benzoate. 4-Methoxy addition to cinnamaldehyde, more than methyl or 2-methoxy additions, likewise increased lure attractiveness to western corn rootworm, producing what is still the most effective single-component lure available for this species (Metcalf and Lampman, 1989a,b; Metcalf and Metcalf, 1992). 4-Methoxy addition to 2-phenyl-1-ethanol likewise enhanced northern corn rootworm attraction in Illinois tests (Metcalf and Lampman, 1991, 1997; Metcalf et al., 1995), but more westerly populations reacted better to 2-phenyl-1-ethanol than to its derivative (Hesler et al., 1994; Hammack, 2003), in common with northern corn rootworm response patterns

seen here with methyl benzoate compared with derivatives formed by functional group additions to its ring.

Cinnamaldehyde and its derivatives are potent diabroticite attractants of cuburbit origin (Metcalf and Metcalf, 1992). Thus, the attractiveness of ethyl *trans*-cinnamate to both northern and western corn rootworm shown here is not surprising, although methyl cinnamate, a widespread floral volatile (Knudsen et al., 1993), was inactive (Metcalf and Lampman, 1989a). Ethyl cinnamate was found in vacuum distillate of maize silks (Flath et al., 1978) and as a sex pheromone constituent of emissions from lepidopteran hairpencils, where floral and fruity odors are common (Nishida et al., 1982).

Some of the relationships explored here between attractant structure and activity will need to be confirmed in additional tests in which compound release rates are equalized and, for the more volatile compounds, maintained for the entire test duration. Release of methyl benzoate, for example, likely fell off before our tests ended, as did that of several other compounds depicted in Figures 3 and 4 and showing nominal release rates greater than about 30 μmoles hr^{-1}. Lure volatilities measured here will facilitate design of these confirmatory studies, although modified dispensers will be needed to slow release of the more volatile lures to realistic levels. Methyl benzoate, for instance, is a common floral volatile (Knudsen et al., 1993) emitted from single blossoms at peak rates near 2 or 12 μg hr^{-1} ($<$0.1 μmoles hr^{-1}) depending on species (Kolosova et al., 2001), rates that are orders of magnitude lower than those achieved here. Another potential problem with the cotton dispensers is binding of compounds to the cotton, a possibility suggested by the slow release of some of the more polar compounds, most notably the nitrogenous ones, relative to their release from aluminum planchets. Dispensers made of inert material would prevent any such binding, but may not change release patterns if the three-dimensional cotton matrix disproportionately slowed release for some of the more polar lures not by binding them but simply by fostering more hydrogen bonding among lure molecules than occurred in the thin film on aluminum. Principles and technologies for dispensing semiochemicals are discussed by Byers (1988) and El-Sayed and Byers (2000).

Vaporization of the most volatile attractants before trapping was terminated likely caused at least one of several discrepancies between results of the 1998 and 2000 tests lasting for 48 and 24 hr, respectively. Greater western corn rootworm captures in 2000 than 1998 on traps baited with methyl benzoate, compared with lower volatility lures such as methyl anthranilate and methyl 4-methoxybenzoate, likely occurred because the former ester would have been released for a proportionately greater portion of the shorter duration test. A similar discrepancy was not evident for northern corn rootworm, but methyl benzoate was the only lure tested in both years that captured high numbers of this species. A difference between results of the two tests that cannot be so readily explained was the significantly greater attractiveness of methyl anthranilate compared with methyl 4-methoxybenzoate

in 2000, but just the opposite pattern in the longer 1998 test. The discoloration of methyl anthranilate in our field trials suggested that degradation in ultraviolet light might account for its lesser efficacy in the 48- than 24-hr test; however, photolysis rate constants, at least those reported for a dilute aqueous solution in simulated sunlight (Aronov and Clark, 1996), are too low to support this explanation.

Methyl benzoate and related benzenoids, like the phenylpropanoids already implicated as an important class of corn rootworm kairomonal attractants (Metcalf et al., 1995), are synthesized *via* the shikimic acid pathway of higher plants. Terpenoid attractants such as β-ionone, linalool, and β-caryophyllene, synthesized via isoprenoid pathways, comprise another important class (Metcalf and Metcalf, 1992; Hammack, 1996, 2001). Shikimic and isoprenoid pathways, along with the octodecanoid pathway yielding green leaf volatiles not yet implicated as corn rootworm lures (Hammack, 2001), produce not only floral aromas (Dudareva and Pichersky, 2000) but also defensive volatiles induced in vegetative tissues by insect herbivory (Kessler and Baldwin, 2001). The latter may prove to be an important functional basis for attractiveness of synthetic lures to diabroticite beetles, despite their known reaction to cucurbit-blossom and maize-silk odors (McAuslane et al., 1986; Prystupa et al., 1988; Metcalf and Metcalf, 1992). Although volatile emissions induced by herbivory typically elicit herbivore damage, such volatiles may attract adult Coleoptera that aggregate on host plants for mating, mass feeding, or sequestration of defensive, secondary plant compounds (Loughrin et al., 1996; Bolter et al., 1997; Kalberer et al., 2001). Sequestration of cucurbitacins, an established activity of diabroticite beetles (Metcalf et al., 1980; Fisher et al., 1984; Eben et al., 1997), may provide a functional basis for such responses to cucurbits, if not to maize. Comparisons of beetle reactions to intact and herbivore-damaged host plants seem warranted for this reason and because of overlap, at least qualitatively, in the composition of floral, host, and herbivore-induced volatiles.

Methyl anthranilate like methyl benzoate occurs among released floral volatiles (Knudsen et al., 1993), and it is also a minor component in the headspace of vegetative maize after insect herbivory (Bernasconi et al., 1998; Turlings et al., 1998). It has been identified as an insect attractant, notably among polyphagous scarabaeid beetles (Imai et al., 1997; Maekawa et al., 1999; Larsson et al., 2003). These scarabaeids, like the diabroticites, respond to a greater or lesser degree to methyl benzoate, methyl salicylate, methyl 2-methoxybenzoate, and methyl 2-methyl benzoate, although not to ethyl or propyl benzoate in the case of the soybean beetle (Maekawa et al., 1999; Larsson et al., 2003). The first two esters, in particular, are widespread floral volatiles (Knudsen et al., 1993), and methyl salicylate is common among defensive chemicals emitted by higher plants, maize included, in response to insect herbivory (Turlings et al., 1998; Kessler and Baldwin, 2001).

Lengthening the side chain of methyl benzoate by one carbon to produce methyl phenyl acetate reduced capture of northern and western corn rootworm beetles in the present study. Such elongation of the phenyl acetate chain, however,

had little effect (benzyl acetate) or, in the case of 2-phenylethyl acetate with two added carbons, significantly enhanced lure attractiveness to both species. Improvement occurred despite reduced volatility and suggested that the activity of 2-phenylethyl acetate did not arise from its structural similarity with methyl benzoate. 2-Phenylethyl acetate, like benzyl acetate, is a common floral volatile that also occurs in the headspace of vegetative maize, where it is a minor constituent above some varieties injured by insect herbivory (Takabayashi et al., 1995; Bernasconi et al., 1998; Turlings et al., 1998; Gouingueni et al., 2001, 2003) but not among herbivore-induced volatiles of the dozen or so other plant species examined to date (Fritzsche Hoballah et al., 2002). Metcalf and Lampman (1991) cited unpublished data showing lesser attractiveness of the acetates of 2-phenyl-1-ethanol and 3-phenyl-1-propanol compared with the corresponding alcohols to northern corn rootworm, but ester activity was not discussed for western corn rootworm nor has it been tested with northern corn rootworms of more westerly origin or in combination with other lures. Phenethyl esters, including 2-phenylethyl acetate, for example, contribute to the efficacy of lure blends attractive to Japanese beetles (Ladd et al., 1973).

Modest synergy has been demonstrated when lures for diabroticite beetles are dispensed in blends (Lampman and Metcalf, 1987; Metcalf et al. 1995, Hammack, 1996, 2001, 2003; Petroski and Hammack, 1998). Lure volatility and release rate data will be needed to decipher not only structure–activity relationships like those examined here but also the complex effects of qualitative and quantitative variation in the composition of multicomponent lures on the host-seeking behavior of diabroticite beetles.

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RELATIVE ATTRACTIVENESS OF (10*E*)-DODECEN-1-YL ACETATE AND (4*E*,10*E*)-DODECADIEN-1-YL ACETATE TO MALE SPOTTED TENTIFORM LEAFMINERS *Phyllonorycter blancardella* (F.)

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Abstract—The antennae of male spotted tentiform leafminers, *Phyllonorycter blancardella*, from Ontario, Canada, exhibited similar electroantennogram responses when stimulated with *E*10-12:Ac or *E*4,*E*10-12:Ac. In field trapping experiments, *E*10-12:Ac was two-fold or more attractive than *E*4,*E*10-12:Ac, and *E*4,*E*10-12:Ac did not enhance the attractiveness of *E*10-12:Ac. *E*4,*E*10-12:Ac has not been identified in the pheromone of *P. blancardella* and it is hypothesized that the structural similarity of this compound and *E*10-12:Ac, the major pheromone compound of this species, may be responsible for the electrophysiological and behavioral responses to *E*4,*E*10-12:Ac. The possible reasons for the disparity between the results of our field trapping experiments and those carried out in Nova Scotia, Canada, and Massachusetts, USA., where *E*4,*E*10-12:Ac was found to be two to four times more attractive to *P. blancardella* than *E*10-12:Ac, are discussed.

Key Words—*Phyllonorycter blancardella*, spotted tentiform leafminer, pheromone, (10*E*)-dodecen-1-yl acetate, (4*E*,10*E*)-dodecadien-1-yl acetate.

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INTRODUCTION

The spotted tentiform leafminer, *Phyllonorycter blancardella* (F.) (Lepidoptera: Gracillariidae), is a European microlepidopteran that was introduced into North America (Pottinger and LeRoux, 1971). Its current distribution in eastern North America is from South Carolina northward to Nova Scotia and westward to Illinois and Ontario; in western North America it has been recorded from Oregon, Washington, and the Vancouver area of British Columbia (Landry and Wagner, 1995). The leafminer's hosts include wild crab apple, *Malus* spp., and apple, *Malus domestica* Borkh (Maier, 1985). It is an important pest in commercial apple orchards in eastern North America and Western Europe (Maier, 1984).

Adult leafminers lay their eggs on the undersurface of apple leaves, and hatching larvae tunnel into the leaf mesophyll where they initially form a serpentine mine. The first three larval instars feed on interstitial fluid, whereas the last two instars consume tissue, which results in a characteristic tent-shaped, white blotch on the leaf surface (Pottinger and LeRoux, 1971). *P. blancardella* has two to five generations per year depending on climate, and overwinters in the pupal stage inside mines on fallen leaves (Pottinger and LeRoux, 1971; Laing and Heraty, 1987).

Roelofs et al. (1977) found that (10*E*)-dodecen-1-yl acetate (*E*10-12:Ac) was a potent sex attractant for *P. blancardella* in New York, U.S.A. apple orchards, but they did not identify this compound in the extracts of the pheromone gland. Mozuraitis et al. (1999) identified *E*10-12:Ac, (10*E*)-dodecan-1-ol (*E*10-12:OH), and dodecan-1-yl acetate (12:Ac) in the extracts of the pheromone gland and effluvia of female *P. blancardella*, although they found no behavioral responses to the latter two compounds in field trapping experiments conducted in Lithuania. In field trapping experiments conducted in Nova Scotia, Canada, and Massachusetts, U.S.A., Gries et al. (1993) found that (4*E*,10*E*)-dodecadien-1-yl acetate (*E*4,*E*10-12:Ac) was two to four times more attractive to *P. blancardella* than *E*10-12:Ac.

In Ontario, Canada, *E*10-12:Ac has been used for monitoring and experimental control of *P. blancardella* (Trimble, 1983, 1984, 1986; Trimble and Hagley, 1988; Trimble and Tyndall, 2000). However, the relative attractiveness of *E*10-12:Ac and *E*4,*E*10-12:Ac has not been compared in this geographic location. We conducted field trapping experiments to investigate the relative attractiveness of *E*10-12:Ac and *E*4,*E*10-12:Ac in an Ontario population of *P. blancardella*. In addition, we compared the relative attractiveness of binary blends containing different ratios and amounts of *E*10-12:Ac and *E*4,*E*10-12:Ac. The responsiveness of male antennae to *E*10-12:Ac and *E*4,*E*10-12:Ac was also compared using the electroantennogram technique.

METHODS AND MATERIALS

Synthesis and Purification of Pheromone Compounds. Liquid chromatography (Baeckström et al., 1987) was performed on silica gel (Merck 60, 0.040–0.063 mm) in 15 or 25-mm inner diam glass columns with gradient elution, using hexane and increasing proportions of ethyl acetate. ^{1}H and ^{13}C NMR spectra of $CDCl_3$ solutions were recorded at 400 and 100 MHz, respectively, with a Bruker AM spectrometer (Bruker BioSpin Ag, Switzerland). GC-MS analyses were performed using a Finnigan SSQ 7000 mass spectrometer (ThermoFinnigan, San Jose, CA, USA), connected to a Varian 3400 GC (Varian Inc., Middelburg, The Netherlands), with electron impact ionization (EI). A DB-5 MS fused silica column (J&W Scientific, 30 m, 0.25 mm-ID, 0.25-μm coating) and a DB-WAX column (J&W Scientific, 30 m, 0.25 mm-ID, 0.25-μm coating layer) were used. Tetrahydrofuran was distilled from sodium/benzophenone ketyl. Commercial starting materials were used without further purification. Reactions involving anhydrous solvent were carried out under an atmosphere of argon.

(10E)-Dodecen-1-yl Acetate. (10E)-Dodecen-1-yl acetate (96% isomeric purity) (Bedoukian Research Inc., Danbury, CT, USA) (1.18 g, 5.25 mmol) was mixed with a hot solution of urea (1.89 g, 31.5 mmol) in methanol (10 ml) (Leadbetter and Plimmer, 1979). The solution was cooled to room temperature and then left in the refrigerator overnight. The resulting crystals were rapidly filtered off, and the solid clathrate was dissolved in brine and extracted with diethyl ether. Drying and concentration gave a mixture (1.00 g; E:Z 98:2). This mixture was subjected to medium pressure liquid chromatography (MPLC), using silica gel impregnated with 10% silver nitrate.

*1,6-Hexane-Bistriphenylphosphonium Dibromide (**2**).* A solution of 1,6-dibromohexane (**1**) (4.88 g, 20.0 mmol) and triphenylphosphine (10.49 g, 40.0 mmol) in 15 ml of dry acetonitrile was stirred at reflux under N_2 for 24 hr. The solvent was evaporated, and the crystals were dried in a vacuum desiccator. The product **2** (90%) was used without further purification. ^{1}H NMR ($CDCl_3$) 7.64–7.82 (m, 30H), 3.69–3.77 (m, 4H), 1.80 (m, 4H), 1.64 (m, 4H). ^{13}C NMR ($CDCl_3$) 134.8, 133.6 (d, $J = 10.2$ Hz), 130.4 (d, $J = 12.4$ Hz), 118.1 (d, $J = 86.2$ Hz), 28.9 (d, $J = 17.0$ Hz), 22.3 (d, $J = 50.2$ Hz).

*2-Hydroxytetrahydrofuran (**3**).* 2,3-Dihydrofuran (50.0 g, 692 mmol) was added dropwise over 20 min to a mixture of concentrated HCl (10 ml) and water (100 ml) at 0°C. The reaction mixture was stirred for 30 min at 0°C, then adjusted to pH 8 with 20% aqueous NaOH solution, and saturated with NaCl. The mixture was extracted with CH_2Cl_2 and the combined extracts were dried over anhydrous $MgSO_4$. Evaporation and distillation provided 2-hydroxytetrahydrofuran in 61% (37.3 g) yield. ^{1}H NMR ($CDCl_3$) 5.27–5.25 (1H, dd, $J = 1.6$ Hz), 3.75–3.65

(2H, m), 1.89–1.6 (4H, m); ^{13}C NMR ($CDCl_3$) 99.33, 66.85, 31.89, 23.14; EIMS m/z 87 $[M\text{-}H]^+$ (3), 72 (4), 71 (100), 70 (3), 43 (22), 41 (19).

*(4E,10E)-Dodecadien-1-ol (E4,E10-12:OH) (**4**).* 1,6-Hexane-bistriphenylphosphonium dibromide (2.12 g, 2.76 mmol) in THF (3.5 ml) was added to a solution of sodium bis-[trimethylsilyl]amide (0.5 M in toluene, 19.56 ml, 9.78 mmol). After refluxing 1 hr, the reaction mixture was cooled to –78°C, and solutions of acetaldehyde (0.11 g, 2.76 mmol) in THF (3.5 ml) and 2-hydroxytetrahydrofuran (0.24 g, 2.76 mmol) in THF (3.5 ml) were added dropwise. The mixture was stirred for 3 hr while it warmed to room temperature, and was then poured into aqueous NH_4Cl (10%, 20 ml). The organic phase was separated and the aqueous phase was extracted with hexane. The combined organic phases were dried over anhydrous $MgSO_4$. After concentration, the crude product was subjected to MPLC, yielding 43% (0.21 g) of a mixture of the four isomers of 4,10-12:OH. The isomeric purity of the *E*4,*E*10-12:OH was increased to >99% by the urea inclusion complex (clathrate) procedure. Thus, the dienol was dissolved in a hot solution of methanol containing 0.2 g urea/ml. Six mol urea were used per mol dienol. The resulting solution was cooled to room temperature and then left in the refrigerator overnight. After filtration and extraction as described above, the recovered dienol was purified by MPLC using $AgNO_3$ impregnated silica gel eluting with: 0%, 1.5%, 2.5%, 5%, 10%, 15%, 30%, 40%, 60%, and 80% ethyl acetate in hexane. ^{1}H NMR ($CDCl_3$) 5.45–5.29 (4H, m), 3.59 (2H, t, J = 6.4 Hz), 2.35 (1H, bs, OH), 2.11–1.90 (6H, m), 1.62–1.54 (5H, m), 1.36–1.28 (4H, m); EIMS m/z 182 $[M]^+$ (0.2), 164 (0.3), 135 (16), 123 (6), 122 (9), 121 (13), 111 (5), 110 (22), 109 (24), 108 (6), 107 (15), 98 (5), 97 (15), 96 (33), 95 (31), 94 (19), 93 (37), 92 (1), 91 (7), 84 (8), 83 (9), 82 (29), 81 (75), 80 (27), 79 (73), 78 (2), 77 (7), 71 (30), 70 (13), 69 (19), 68 (96), 67 (100), 66 (9), 65 (6), 57 (8), 56 (5), 55 (72), 54 (18), 53 (20), 43 (10), 42 (5), 39 (22), 31 (11).

*(4E,10E)-Dodecadien-1-yl Acetate (E4,E10-12:Ac) (**5**).* *E*4,*E*10-12:OH (180 mg, 0.99 mmol) was stirred in acetic anhydride and pyridine (0.6 ml, 1:2 v/v) at 0°C for 1 hr and at room temperature for 2 hr. The reaction mixture was poured into ether and washed with water (2 × 25 ml), the aqueous phase was extracted with ether, and the combined organic phases were dried ($MgSO_4$). The yield after chromatography with the same solvent system used for purification of compound **4** was 200 mg (89%) and the isomeric purity was >99%. ^{1}NMR ($CDCl_3$) 5.45–5.35 (4H, m), 4.05 (2H, t, J = 6.8 Hz), 2.08–2.01 (2H, m), 2.03 (3H, s), 2.0–1.92 (4H, m), 1.67 (2H, quint, J = 6.8 Hz), 1.64–1.62 (3H, m), 1.36–1.29 (4H, m); ^{13}C NMR ($CDCl_3$) 171.13, 131.41 (2C), 128.68, 124.64, 64.0, 32.39, 31.56, 29.1, 29.08, 28.82, 28.44, 20.94, 17.86.

Electroantennogram. Apple leaves infested with *P. blancardella* were collected from an orchard at the Agriculture and Agri-Food Canada Experimental Farm in Jordan Station, Ontario. Pupae were removed, sexed (Pottinger and LeRoux, 1971), and male pupae were held in 33 cm × 33 cm × 33 cm

polycarbonate cages for adult emergence. Antennal responses were measured with a Syntech® High-Resistance EAG Probe, Type ID-02 Signal Interface Box and Type IDAC-02 Intelligent Data Acquisition Controller (Syntech, Hilversum, The Netherlands). Syntech® electroantennogram software was used to filter, amplify, visualize, and store antennal response data on a personal computer. The antennae of 2- to 3 d-old males were excised at their bases and attached to the electrodes of the probe using Spectra® 360 Electrode Gel (Parker Laboratories, Inc., Orange, NJ). Several segments were removed from the distal end of the antenna to facilitate conductivity. Each antennal preparation was continuously exposed to a humidified and charcoal-filtered airstream (0.5 l/min) supplied through a glass tube (6 mm-ID, 9 mm-OD). Four doses (1, 10, 100 or 1000 ng) of *E*10-12:Ac and *E*4,*E*10-12:Ac were tested. Test compounds were applied to a 1 cm^2 piece of Whatman® No. 1 filter paper (Whatman International Ltd., Maidstone, England) in 10 μl of HPLC-grade hexane. Ten μl of hexane were applied to a filter paper as a control. The filter papers were placed in Pasteur pipettes after the solvent had evaporated. Each antenna was first stimulated with the control, and then with increasing concentrations of one compound. The stimuli were delivered as 0.5-sec pulses at an air flow rate of 0.5 ml/min using a Syntech® Type CS-01 Stimulus Controller. A 60-sec recovery period followed application of a stimulus. Pasteur pipettes and filter paper squares were renewed after 4 hr of use. The four concentrations of one compound were administered to each of five antennae. Linear regression analysis was used to describe the relationship between antennal response and stimulus concentration (SAS Institute Inc., 1998). The significance of differences in the slope and intercept of the antennal response-stimulus concentration relationship for each compound was tested using analysis of covariance (SAS Institute Inc., 1998).

Field Trapping Experiments. The relative attractiveness of different amounts and ratios of *E*10-12:Ac and *E*4,*E*10-12:Ac was compared during the second flight (9–25 July 2002) of *P. blancardella* in apple orchards at the Agriculture and Agri-Food Canada Experimental Farm in Jordan Station, Ontario. In the first experiment five treatments were compared: (1) 1.0-μg *E*10-12:Ac, (2) 1.0-μg *E*10-12:Ac + 0.1-μg *E*4,*E*10-12:Ac, (3) 1.0-μg *E*10-12:Ac + 1.0-μg *E*4,*E*10-12:Ac, (4) 0.1-μg *E*10-12:Ac + 1.0-μg *E*4,*E*10-12:Ac, and (5) 1.0-μg *E*4,*E*10-12:Ac (i.e. experiment of Gries et al., 1993). In the second experiment, five ratios of the two compounds were compared: (1) 1.0-μg *E*10-12:Ac, (2) 0.9-μg *E*10-12:Ac + 0.1-μg *E*4,*E*10-12:Ac, (3) 0.5-μg *E*10-12:Ac + 0.5-μg *E*4,*E*10-12:Ac, (4) 0.1-μg *E*10-12:Ac + 0.9-μg *E*4,*E*10-12:Ac, and (5) 1.0- μg *E*4,*E*10-12:Ac. In the third experiment, four doses of each compound were compared: (1) 0.1-μg *E*10-12:Ac, (2) 1.0-μg *E*10-12:Ac, (3), 10-μg *E*10-12:Ac (4), 100-μg *E*10-12:Ac, (5) 0.1-μg *E*4,*E*10-12:Ac, (6) 1.0-μg *E*4,*E*10-12:Ac, (7), 10-μg *E*4,*E*10-12:Ac (8), 100-μg *E*4,*E*10-12:Ac. Compounds were applied to the large "well" of 9-mm diam, natural rubber sleeve stoppers (Chromatographic

Specialities, Brockville, Ontario) in 200 μl of analytical grade hexane (Fisher Scientific, Ottawa, Ontario). The solvent was allowed to evaporate in a fume hood, and the stoppers were stored until use at –20°C. A stopper treated with 200 μl of hexane was included in each experiment as a control. The treatments were compared using a randomized complete block design (Snedecor and Cochran, 1967). Five trap lines were established at about 20-m intervals in an orchard and five or six "trapping stations" were established at about 20-m intervals in each trap line. White plastic delta traps (Cooper Mill Ltd., Madoc, Ontario) each containing a pheromone-impregnated stopper were placed approximately 1.5–2.0 m from the ground within a tree at each trapping station. Each of the five or six treatments was randomly assigned to a trapping station within a trap line. Traps were exposed for 3–5 d. The effect of treatment on the mean total number of moths captured was tested using ANOVA (SAS Institute Inc., 1998) after variances were stabilized using the $\sqrt{x}$ transformation. Significantly different treatment means were identified using Fisher's Protected Least Significant Difference Test (SAS Institute Inc., 1998).

Leafminer Identification. Two percent of the leafminers captured in each trap were randomly removed and identified using features of their genitalia as described by Landry and Wagner (1995).

RESULTS

(4E,10E)-Dodecadien-1-yl Acetate. E4,E10-12:OH, 4, was produced by a mixed Wittig reaction of acetaldehyde and the cyclic hemiacetal 2-hydroxytetrahydrofuran (Bestmann et al., 1978) with the ylide prepared from 1,6-hexanebistriphenylphosphonium dibromide, using NaHMDS as base (Figure 1). A mixture of geometrical isomers of 4,10-dodecadien-1-ols was obtained. Although the Z,Z-isomer was probably the major isomer in the product mixture, the E,E-isomer was selectively extracted as a urea inclusion complex (clathrate) (Leadbetter and Plimmer, 1979). This process was repeated several times to increase the yield of $E4,E10$-12:OH to >180 mg. The isomeric purity of the $E4,E10$-12:OH was then increased to >99% by MPLC on $AgNO_3$ impregnated silica gel (Vanbeek and Subrtova, 1995). Acetylation of $E4,E10$-12:OH, **4**, in pyridine and acetic anhydride yielded acetate **5** (89%). No loss of isomeric purity was observed.

Electroantennogram Tests. There was a linear relationship between electroantennogram response (mV) and the common $\log_{10}$ dose (ng) of $E10$-12:Ac ($y = 0.117 + 0.138[\log_{10}x]$, $F = 10.6$, df = 1, 18, $P = 0.005$, $r^2 = 0.37$) and $E4,E10$-12:Ac ($y = 0.060 + 0.096[\log_{10}x]$, $F = 70.0$, df = 1, 18, $P < 0.001$, $r^2 = 0.79$) used in the stimulus delivery system (Figure 2). The slope of the electroantennogram dose-response relationship for each compound was similar ($F = 0.91$, df = 1, 36, $P = 0.35$). However, the intercept of the dose-response

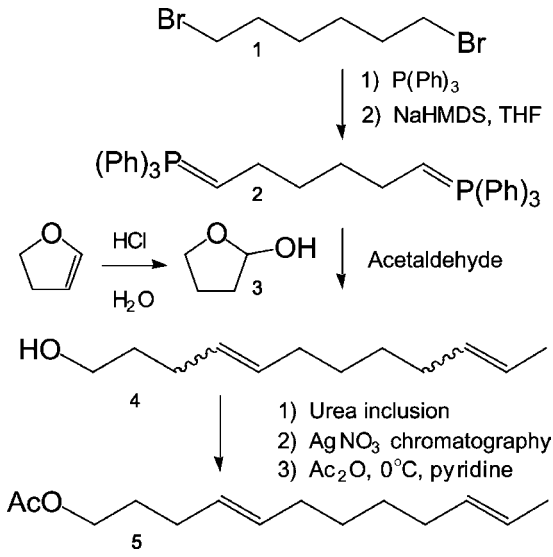

FIG. 1. Synthesis of (4*E*,10*E*)-dodecadien-1-yl acetate.

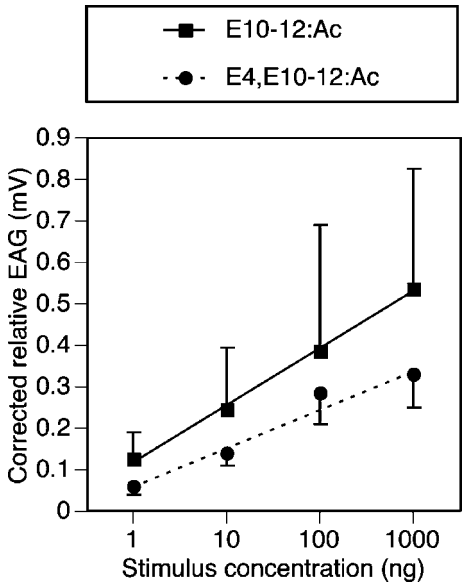

FIG. 2. Electroantennogram responses of male *Phyllonorycter blancardella* antennae to (10*E*)-dodecen-1-yl acetate and (4*E*,10*E*)-dodecadien-1-yl acetate.

relationship for E10-12:Ac was greater than the intercept of the relationship for $E4,E$10-12:Ac ($F = 5.96$, df $= 1, 37$, $P = 0.02$).

Field Trapping Experiments. In the repetition of the Gries et al. (1993) experiment, lures loaded with 1.0 μg of E10-12:Ac were 2.5 times more attractive than lures loaded with 1.0 μg of $E4,E$10-12:Ac. The attractiveness of lures loaded with 1.0 μg E10-12:Ac was not enhanced by the addition of 0.1 or 1.0 μg of $E4,E$10-12:Ac ($F = 214.43$, df $= 5, 20$, $P < 0.001$) [Table 1, Experiment 1]. The attractiveness of 1.0 μg of pheromone declined as the amount of $E4,E$10-12:Ac increased further ($F = 95.79$, df $= 5, 20$, $P < 0.001$) [Table 1, Experiment 2]. The attractiveness of E10-12:Ac was 79 times greater than $E4,E$10-12:Ac when the compounds were compared at 0.1 μg, 12.4 times greater when compared at 1.0 μg, and 3.1 times greater when compared at 10 μg. The compounds had

TABLE 1. RELATIVE ATTRACTIVENESS OF LURES CONTAINING DIFFERENT AMOUNTS OF TWO SYNTHETIC PHEROMONE COMPOUNDS TO MALE *Phyllonorycter blancardella*

Amount of compound	Mean (±SD) total number captured per trap
Experiment 1	
1.0-μg E10-12:Ac	88.0 ± 41.6a
1.0-μg E10-12:Ac + 0.1-μg $E4,E$10-12:Ac	96.2 ± 13.0a
1.0-μg E10-12:Ac + 1.0-μg $E4,E$10-12:Ac	101.2 ± 25.1a
0.1-μg E10-12:Ac + 1.0-μg $E4,E$10-12:Ac	49.4 ± 19.2b
1.0-μg $E4,E$10-12:Ac	34.8 ± 10.1b
Control	0.0 ± 0.0c
Experiment 2	
1.0-μg E10-12:Ac	63.8 ± 23.6a
0.9-μg E10-12:Ac + 0.1-μg $E4,E$10-12:Ac	67.6 ± 25.2a
0.5-μg E10-12:Ac + 0.5-μg $E4,E$10-12:Ac	29.2 ± 12.8b
0.1-μg E10-12:Ac + 0.9-μg $E4,E$10-12:Ac	13.6 ± 2.8c
1.0-μg $E4,E$10-12:Ac	16.0 ± 8.6c
Control	0.0 ± 0.0d
Experiment 3	
0.1-μg E10-12:Ac	27.8 ± 29.0c
1.0-μg E10-12:Ac	57.2 ± 36.3c
10.0-μg E10-12:Ac	154.8 ± 78.2b
100.0-μg E10-12:Ac	380.2 ± 102.6a
0.1-μg $E4,E$10-12:Ac	0.4 ± 0.5e
1.0-μg $E4,E$10-12:Ac	4.6 ± 5.1d
10.0-μg $E4,E$10-12:Ac	49.6 ± 28.2c
100.0-μg $E4,E$10-12:Ac	216.4 ± 103.7ab
Control	2.2 ± 3.5de

Note. Within each experiment, means in a row followed by the same letter are not significantly different (Fisher's Protected Least Significant Difference test, $P > 0.05$).

similar attractiveness when compared at a concentration of 100 μg ($F = 40.93$, df = 8, 32, $P < 0.001$) [Table 1, Experiment 3].

Leafminer Identification. Two of the 148 identified leafminers (1.4%) were *Phyllonorycter mespilella* (Hübner), and the remainder were *P. blancardella*. One *P. mespilella* was found in a trap baited with 1.0-μg *E*10-12:Ac + 0.1-μg *E*4,*E*10-12:Ac (Experiment 1, Treatment 2), and the other was found in a trap baited with 100.0-μg *E*4,*E*10-12:Ac (Experiment 3, Treatment 8).

DISCUSSION

The antennae of male *P. blancardella* from Ontario exhibited similar electroantennogram responses when stimulated with *E*10-12:Ac and *E*4,*E*10-12:Ac, but in field trapping experiments, *E*10-12:Ac was twofold or more attractive than *E*4,*E*10-12:Ac, and *E*4,*E*10-12:Ac did not enhance the attractiveness of *E*10-12:Ac. *E*4,*E*10-12:Ac has not been identified in the pheromone of *P. blancardella* (Roelofs et al., 1977; Mozuraitis et al., 1999; El-Sayed et al., unpublished data), and, therefore, the structural similarity of this compound and *E*10-12:Ac may be responsible for the antennal and behavioral responses to *E*4,*E*10-12:Ac in the electroantennogram and field trapping experiments, respectively. Pheromone analogues have been observed to stimulate both electroantennogram and behavioral activity in other Lepidoptera (Schwarz et al., 1990).

In contrast to the results of field trapping experiments carried out in Nova Scotia, Canada, and Massachusetts, USA, where *E*4,*E*10-12:Ac was found to be two to four times as attractive to *P. blancardella* as *E*10-12:Ac (Gries et al., 1993), we found that in Ontario, *E*10-12:Ac was twofold or more attractive than *E*4,*E*10-12:Ac. There are several possible reasons for the disparity between our results and those of Gries et al. (1993). First, a related species, *Phyllonorycter mespilella* (Hübner) was not known to occur in eastern North America (Landry and Wagner, 1995), and it may have been mistaken for *P. blancardella*. *E*4,*E*10-12:Ac has been identified as a pheromone component of *P. mespilella* and was attractive to this species in field trapping experiments (Gries et al., 1993). The two *P. mespilella* that we identified in samples of leafminers captured in Ontario were from traps baited with *E*4,*E*10-12:Ac as a single component or part of a blend. A second possible reason for the disparity between our results and those of Gries et al. (1993) may be geographic variation in the pheromone chemistry of *P. blancardella*. Polymorphic variation is known in the Lepidoptera, with different populations sometimes utilizing different compounds or ratios in their pheromones (Löfstedt, 1990). For example, in the larch budmoth, *Zeiraphera diniana* Guenée (Lepidoptera: Tortricidae), one race feeds on larch, *Larix decidua* Miller, and utilizes (11*E*)-tetradecen-1-yl acetate (*E*11-14:Ac) for male attraction, whereas another race feeds on cembrian pine, *Pinus cembra* L., and

utilizes (9*E*)-dodecen-1-yl acetate (*E*9-12:Ac) for male attraction (Guerin et al., 1984).

The lack of any apparent synergism between *E*10-12:Ac and *E*4,*E*10-12:Ac in our field trapping experiments could be due to the fact that *E*4,*E*10-12:Ac has no role in the mate seeking behavior of *P. blancardella*, or optimal ratios of the two compounds were not tested. In our experiments, binary mixtures contained 10% or more of *E*4,*E*10-12:Ac, whereas in many species of Lepidoptera synergism occurs when secondary compounds are present in trace amounts relative to the main pheromone compound (Arn et al., 1992; El-Sayed, 2003). In a subsequent study, lower ratios (i.e., <10%) of *E*4,*E*10-12:Ac were tested with no apparent effect on male attraction (El-Sayed et al., unpublished data), suggesting that this compound has no role in the mate seeking behavior of this species.

The relatively weak behavioral responses stimulated by *E*4,*E*10-12:Ac and the lack of synergism with *E*10-12:Ac in an Ontario population of *P. blancardella* does not rule out the possibility that *E*4,*E*10-12:Ac is involved in sex attraction of other North American populations of this species. The field trapping experiment of Gries et al. (1993) should be repeated in Nova Scotia and Massachusetts to rule out the possibility that *P. mespilella* may have been misidentified as *P. blancardella*. In addition, the relative attractiveness of *E*10-12:Ac and *E*4,*E*10-12:Ac to *P. blancardella* should be compared in other North American locations to determine whether this species exhibits polymorphic variation in the chemistry of its pheromone.

The results of our trapping experiments demonstrate that *E*10-12:Ac is more attractive to *P. blancardella* than *E*4,*E*10-12:Ac, and, therefore, *E*10-12:Ac should be used in lures for monitoring the occurrence and activity of this species in Ontario. Our results also demonstrate the occurrence of the related species *P. mespilella* in Ontario, and although this species was captured only in traps baited with *E*4,*E*10-12:Ac, samples of leafminers trapped in this location should be examined to confirm their presumed identity.

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BEHAVIORAL ACTIVITY OF STEREOISOMERS AND A NEW COMPONENT OF THE CONTACT SEX PHEROMONE OF FEMALE GERMAN COCKROACH, *Blattella germanica*

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Abstract—(3*S*,11*S*)-3,11-Dimethylnonacosan-2-one is a major component of the courtship stimulating, contact sex pheromone of the female German cockroach. Although the four synthetic stereoisomers of this compound have been tested in behavioral assays, their relative activity remains unresolved. Using isolated male antennae dosed with synthetic test compounds to assay male behavior, we found that at high doses all four stereoisomers elicited responses from 100% of the males. However, at physiologically relevant doses similar to those found on the female antenna, the (3*S*,11*S*)-isomer was the least effective of the four stereoisomers at eliciting courtship responses in males. This is the first example of a natural stereoisomer having less bioactivity than related stereoisomers that do not occur naturally. Another component of the sex pheromone blend, 3,11-dimethylheptacosan-2-one, was previously purified from the female's epicuticule and behaviorally assayed, but its activity was not confirmed through synthesis. We now confirm that synthetic (3*S*,11*S*)-3,11-dimethylheptacosan-2-one elicits behavioral responses, but less so than its C_{29} homolog.

Key Words—Contact sex pheromone, stereoisomers, German cockroach, behavioral assay, 3,11-dimethylnonacosan-2-one, 3,11-dimethylheptacosan-2-one.

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INTRODUCTION

Upon contact with a mature female, sexually mature males of the German cockroach, *Blattella germanica* (L.), exhibit characteristic courtship behavior that includes an unmistakable raising of the wings and turning away from the female (Roth and Willis, 1952; Nishida et al., 1974; review: Gemeno and Schal, 2004). This act exposes specialized tergal glands on the 7th and 8th abdominal segments of the male, which in turn attract the female to mount the male's abdomen. As the female feeds on nutrients within reservoirs of the tergal glands, she is properly aligned in a precopulatory position (Nojima et al., 1999). The elicitor of this behavioral sequence is a contact sex pheromone blend composed of several oxygenated derivatives of methyl-branched cuticular hydrocarbons. The most abundant component is 3,11-dimethylnonacosan-2-one (3,11-diMeC_{29}-2-one), while two other components, 29-hydroxy-3,11-dimethylnonacosan-2-one and 29-oxo-3,11-dimethylnonacosan-2-one (review: Nishida and Fukami, 1983) are presumably derived from it. The stereochemistry of the natural pheromone was suggested as 3*S*, by comparison of its optical rotation to that of a methyl ketone with an authentic *S*-configuration, together with its NMR measurement in the presence of a chiral shift reagent (Nishida et al., 1974). Its 11*S*-stereochemistry could be rigorously determined only after the synthesis of all four stereoisomers of 3,11-dimethylnonacosan-2-one, followed by comparison of their infrared spectra (as crystals in KBr disks, not as solutions), specific rotations, and melting points to those of the natural pheromone. 500 MHz ^{1}H or 125 MHz ^{13}C-NMR comparisons were useless, all the isomers having shown identical spectra. The final proof of the (3*S*,11*S*)-stereochemistry of the natural pheromone rested on the observed lack of melting point depression (mp 44–45°C) in a mixed melting point determination of the (3*S*,11*S*)-isomer (mp 44–44.5°C) with the natural pheromone (mp 45–46°C), whereas the mixtures of each of the remaining three stereoisomers with the natural pheromone melted at the range between 33.5–37.5°C (Mori et al., 1981).

Synthesis of the four stereoisomers of 3,11-diMeC_{29}-2-one (Mori et al., 1981) enabled behavioral assays of their relative activity. Unlike the high degree of stereospecificity demonstrated in the antennae of many insects, in which unnatural stereoisomers are usually less active, or even have an antagonistic effect, the four stereoisomers of 3,11-diMeC_{29}-2-one were shown to be equally active (review: Nishida and Fukami, 1983). However, Abed et al. (1993) reported preliminary observations (data were not presented) that at low doses the (3*S*,11*S*)-isomer was more active than the others. The latter assays used higher purity stereoisomers synthesized by Mori and Takikawa (1990). In the present work we conducted dose-response behavioral assays with the four stereoisomers of 3,11-diMeC_{29}-2-one of Mori and Takikawa (1990), in an effort to resolve this discrepancy.

A putative fourth pheromone component, 3,11-dimethylheptacosan-2-one (3,11-diMeC_{27}-2-one), was purified by gas chromatography (GC) from female

cuticular lipids and shown to be behaviorally active (Jurenka et al., 1989; Schal et al., 1990). Its gross structure was confirmed by synthesis and mass spectral comparison (Takikawa et al., 1997). We now report on the biological activity of synthetic (3*S*,11*S*)-3,11-diMeC_{27}-2-one (Takikawa et al., 1997) and confirm its activity as a component of the sex pheromone.

METHODS AND MATERIALS

Insects. *Blattella germanica* cockroaches were kept in groups at 27°C under 12:12 light–dark photoperiod and fed dry Purina rat chow and water. Newly emerged adult males and females were separated daily from collectively reared nymphs. Wild cockroaches were collected with a modified vacuum cleaner from an infested commercial pig farm in Warsaw, NC. Adult males were isolated from the collection at least 3 d prior to using them in behavioral assays.

Chemicals and Bioassays. The four stereoisomers of 3,11-diMeC_{29}-2-one were synthesized by Mori and Takikawa (1990) and had >99% diastereomeric excess and ≈100% enantiomeric excess. (3*S*,11*S*)-3,11-Dimethylheptacosan-2-one was synthesized by Takikawa et al. (1997). Each compound was dissolved in hexane, and the concentration of each stock solution was confirmed by GC (HP5890II, HP-5 column 30 m × 0.25 mm × 0.25 μm, splitless injection) relative to *n*-hexacosane as internal standard.

Male behavioral responses were tested using a modification of the "antenna on a stick" assay developed by Roth and Willis (1952). An antenna of a 14–21 d-old adult male *B. germanica* was excised, attached to a glass Pasteur pipette, and either extracted briefly in hexane to remove male cuticular lipids prior to application of the test compound, or used fresh. A 3-μl hexane solution of a test compound was then applied to the distal 1 cm of the test antenna. The hexane was allowed to evaporate, and the antenna was used immediately to test the responses of 30 males or several groups of 10 males 14–21 d old that were housed individually in 8-cm-ID × 8-cm-deep plastic cages supplied with rat chow and water. All assays were conducted during mid-scotophase, avoiding the first and last 2 hr of the scotophase. Each individual male was tested sequentially. The antennae of each male were gently stroked with the test antenna for up to 1 min, and a positive response was recorded when the male executed a courtship response, rotating his body relative to the stimulus and raising his wings within 1 min. This is an unmistakable response that occurs only in a sexual context and is never elicited by male test antennae unfortified with female pheromone or treated with hexane alone.

Amount of Pheromone on Female Antennae. Antennae from ten 5-d-old females were extracted in hexane containing 100 ng of internal standard (heptacosan-14-one). The base of each antenna was prevented from contact with hexane so no

internal lipids were extracted. Five groups of 10 paired antennae were extracted. The extracts were reduced under N_2 to 1 μl and injected into a capillary GC column, as above. The GC oven temperature was kept at 70°C for 1 min, then elevated 30°C per min to 150°C, and 10°C per min to 300°C. The amount of pheromone per antenna was calculated by comparison of the area of the pheromone peak to that of the internal standard.

Statistical Analysis. Dose response assays were analyzed using chi-square analysis to find a discriminating dose and pairwise chi-square comparison with Fisher's exact test within the discriminating dose. ANOVA was used to find differences in responses to various compounds at a single dose. All statistical analysis was performed with SAS (SAS Institute, 2000).

RESULTS AND DISCUSSION

Amount of Pheromone on Female Antennae. Males generally orient to the female's antennae before performing courtship. GC-FID analyses indicated that each antenna of 5-d-old adult females contained 0.99 $\pm$ 0.12 ng (SEM, $N = 5$) of 3,11-diMeC_{29}-2-one, the major component of the contact sex pheromone, and 0.409 $\pm$ 0.004 ng of 3,11-diMeC_{27}-2-one. It is important to note, however, that female antennae also contain minute amounts of other, more active pheromone components, including the 29-oxo- and 29-hydroxy-analogs of 3,11-diMeC_{29}-2-one. Moreover, by touching other parts of the female body with his antennae, a male would be exposed to as much as 250 μg and 100 μg of 3,11-diMeC_{29}-2-one and 3,11-diMeC_{27}-2-one, respectively (Schal et al., 1990).

(3S,11S)-3,11-Dimethylheptacosan-2-one. The synthetic (3*S*,11*S*)-3,11-diMeC_{27}-2-one elicited little response from males at 1 ng, but 100% of tested males exhibited courtship responses at doses $\geq$10 ng (Figure 1A). This result confirms that 3,11-diMeC_{27}-2-one is indeed a component of the sex pheromone of *B. germanica* and corroborates previous results showing that natural 3,11-diMeC_{27}-2-one was less active than the C_{29} methyl ketone (Schal et al., 1990). However, these results indicate that the amount of 3,11-diMeC_{27}-2-one on the female antennae is insufficient by itself to elicit the full sexual response in males. Rather, they indicate that for contact with the antennae alone to elicit the full courtship response, 3,11-diMeC_{27}-2-one would need to operate in concert with the C_{29} methyl ketone pheromone, and possibly other components.

3,11-DiMeC_{29}-2-one is derived from the major cuticular hydrocarbon 3,11-dimethylnonacosane (Chase et al., 1990; Schal et al., 2003). The other pheromone components, 29-oxo- and 29-hydroxy-C_{29}, are probably derived, in turn, from 3,11-diMeC_{29}-2-one. Because 3,11-dimethylheptacosane is also a component of the cuticular lipids, it is probable that 3,11-diMeC_{27}-2-one is derived from it. If

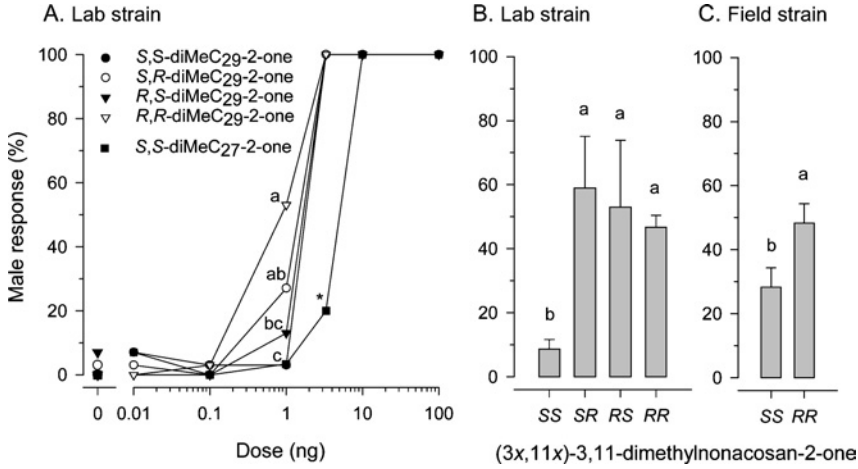

FIG. 1. Dose-behavioral response assays of the sexual responses of *Blattella germanica* adult males to four stereoisomers of 3,11-dimethylnonacosan-2-one and to (3*S*,11*S*)-3,11-dimethylheptacosan-2-one. In A, 30 males from a lab culture were assayed at each dose, whereas in B, a discriminating dose of 1 ng was used to assay three groups of 30 males each. In C, six groups of 10 males each, collected from a field population, were assayed with two of the stereoisomers. In A, different letters indicate significant differences based on χ^2 2×2 test of independence with Fisher's exact test; at all other doses there were no significant differences among treatments. Asterisk (*) indicates significantly higher responses to (3*S*,11*S*)-3,11-dimethylnonacosan-2-one than to (3*S*,11*S*)-3,11-dimethylheptacosan-2-one, at 3 ng. In B and C, different letters indicate significant differences based on ANOVA; SEM is shown for each mean.

so, it is possible that the C_{27} methyl ketone also gives rise to oxidation derivatives that might be present in the pheromone mixture. Because 29-hydroxy-3,11-diMeC$_{29}$-2-one is ~10-fold more active than the methyl ketone (Nishida et al., 1976), and 29-oxo-3,11-diMeC$_{29}$-2-one has intermediate activity between these two components (Nishida and Fukami, 1983), it is reasonable to expect that the C_{27} derivatives, if found, will show structure-activity patterns similar to the C_{29} pheromone components.

The stereochemistry of the naturally occurring 3,11-diMeC$_{27}$-2-one has not been established, and because no stereoselectivity was found in the male German cockroach for the major pheromone component, 3,11-diMeC$_{29}$-2-one (Figure 1A), bioactivity of the *S*,*S*-isomer does not necessarily demonstrate that it is the natural isomer. Nevertheless, it is reasonable to infer based on the (3*S*,11*S*) configuration of the other pheromone components that the 3*S*,11*S* configuration is most probable for 3,11-diMeC$_{27}$-2-one as well (Takikawa et al., 1997).

Stereoisomers of 3,11-Dimethylnonacosan-2-one. All four stereoisomers of 3,11-diMeC$_{29}$-2-one exhibited sharp dose-response curves ranging from background responses to 0.1 ng that were no different from responses to the solvent control, to 100% male response to 3 ng (Figure 1A). Surprisingly, however, these dose-response bioassays with carefully calibrated solutions showed that the *S*,*S*-isomer of 3,11-diMeC$_{29}$-2-one was significantly less active than the other three isomers (Figure 1A). This was unexpected because the *S*,*S*-isomer represents the natural configuration of 3,11-diMeC$_{29}$-2-one. Nevertheless, this observation was further confirmed with independent assays using freshly calibrated standard solutions and a discriminating dose of 1 ng loaded on the test antenna; again, 3*S*,11*S*-diMeC$_{29}$-2-one was significantly less active than the other three isomers (Figure 1B). This discriminating dose, interestingly, is similar to what is naturally found on a female antenna; and yet, a female antenna rarely fails to elicit courtship behavior in mature males. Therefore, either other pheromonal components are crucial for eliciting this high response, or the female antenna possesses textural/mechanical features that elicit higher sexual responses.

A third independent confirmation was obtained when activity of two of the synthetic isomers, *R*,*R*- and *S*,*S*-, was compared to the natural 3*S*,11*S*-diMeC$_{29}$-2-one that was extracted and purified from females by Nishida et al. (1974). The results are shown in Table 1. Male responses to the natural and synthetic 3*S*,11*S*-diMeC$_{29}$-one were similar, but significantly lower (ANOVA, $P < 0.05$) than to synthetic 3*R*,11*R*-diMeC$_{29}$-2-one.

It is possible that these unexpected results were an artifact of working with a cockroach colony that has been maintained in the laboratory for several decades. Therefore, we tested the *S*,*S*- and *R*,*R*-isomers of 3,11-diMeC$_{29}$-2-one on males that were freshly collected from an infestation in the field (Figure 1C). The results of this fourth assay were consistent with our previous findings that the natural stereoisomer was significantly less bioactive than one of the unnatural isomers, *R*,*R*.

In contrast, previous assays showed no significant differences in the doses of the four stereoisomers that were required to elicit courtship responses in males (Nishida et al., 1979; Nishida and Fukami, 1983). These differences might be

TABLE 1. MALE SEXUAL RESPONSES TO MALE ANTENNAE LOADED WITH NATURAL OR SYNTHETIC FEMALE SEX PHEROMONE

Compound tested (2 ng)	Males responding (%)[a]
Natural (3*S*,11*S*)-3,11-dimethylnonacosan-2-one	20.7 ± 4.7 a
Synthetic (3*S*,11*S*)-3,11-dimethylnonacosan-2-one	22.0 ± 3.3 a
Synthetic (3*R*,11*R*)-3,11-dimethylnonacosan-2-one	45.0 ± 7.2 b

[a] Fourteen groups of 10 males each were tested. SEM is shown for each mean. Different letters indicate significant differences ($P < 0.05$) based on ANOVA.

due to methodological differences. For example, the enantiomeric purity of the starting material [(*R*)-citronellic acid] used to synthesize the four stereoisomers in the early studies was ≈92% e.e. Our bioassays used stereoisomers from a more recent synthesis that used enantiomerically pure (*R*)-citronellol, which resulted in exceptionally pure stereoisomers, especially after careful recrystallization of both the intermediates and the final products (Mori and Takikawa, 1990). Also, Nishida and colleagues used antennae of the cockroach *Supella longipalpa* as a substrate for testing pheromone isomers and analogs, whereas we used antennae from conspecific *B. germanica* males. We observed that *S. longipalpa* antennae have some, albeit low, endogenous courtship-stimulating activity on *B. germanica* males (D. Eliyahu, preliminary data).

It is also possible that *B. germanica* male antennae have a compound(s) that masks or inhibits the response to the female pheromone. For example, Nishida and Fukami (1983) found that certain fatty acids inhibit the sexual responses of males to the contact pheromone. In *Nauphoeta cinerea*, the lobster cockroach, a male-specific pheromone (nauphoetin, octadecyl (*Z*)-9-tetracosenoate) elicits aggressive antennal fencing among males, but also serves as a courtship depressant (Fukui and Takahashi, 1983). Our results might, therefore, be explained if such a compound occurs in *B. germanica*, and if it more specifically inhibits the courtship response to the natural isomer than to the other isomers. To test this hypothesis, dose-response experiments were conducted with male test antennae that were extracted briefly in hexane prior to application of the test compound. These antennae were compared to fresh antennae that were not extracted before application (Figure 2). Although pre-extracting the antennae tended to reduce male responses, these depressions were not statistically significant at either low (0.1 and 1 ng) or high doses (10 and 100 ng). This general tendency was likely due to the stiffer and more brittle nature of the test antenna after hexane extraction. However, at a dose of 3.3 ng, extracted antennae that were loaded with the *S*,*R*- and *R*,*S*-pheromone stereoisomers stimulated courtship in significantly fewer males than fresh antennae loaded with the same stereoisomers, respectively. Although the same pattern was evident with the *R*,*R*- and *S*,*S*-stereoisomers, these minor differences were not statistically significant. At any rate, the dose-response studies showed, in a fifth independent test, that a synthetic stereoisomer of the natural pheromone (3*S*,11*S*) was the least bioactive of the four synthetic stereoisomers (Figure 2).

Interestingly, Abed et al. (1993) used the same high-purity stereoisomers that were synthesized by Mori and Takikawa (1990), but they suggested that 3*S*,11*S*-diMeC$_{29}$-2-one was more active than other stereoisomers. Resolution of this discrepancy will have to await publication of the methods and data in support of the brief mention of these preliminary results by Abed et al. (1993).

The importance of stereochemistry in olfactory communication is well known. Pheromones may occur as stereoisomeric mixtures, in which case each isomer may be independently active or the mixture may be most active (Mori,

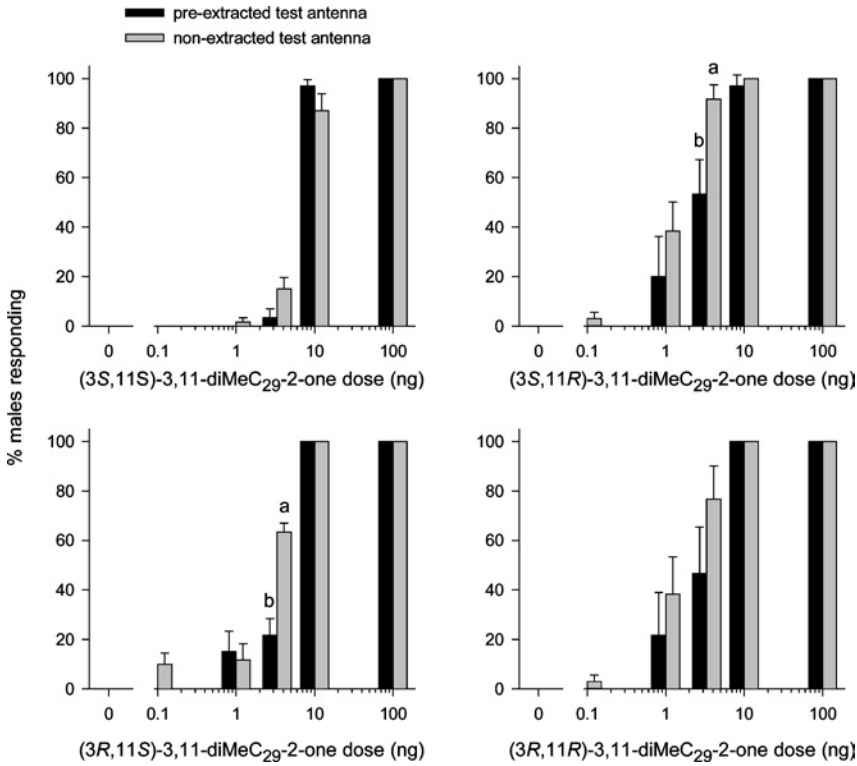

FIG. 2. Dose behavioral response assays of the sexual responses of *Blattella germanica* adult males to four stereoisomers of 3,11-dimethylnonacosan-2-one, applied onto extracted, or fresh nonextracted male antennae. For the doses of 1 and 3.3 ng, six groups of 10 males each were tested with each antennal treatment for responses to each of the four stereoisomers; three groups of 10 males each were tested with the other doses. SEM is shown for each mean. Different letters indicate significant differences based on ANOVA.

1998). In most cases, however, production of the pheromone is stereospecific, and so is its reception in the opposite sex, with the natural pheromone isomer being the most bioactive. Indeed, in closely related, sympatrically occurring species, the nonnatural isomer may have antagonistic effects on sexual orientation, as a mechanism of precopulatory reproductive isolation (Millar, 2000). Nevertheless, insects exhibit a wide range of relations between pheromone stereochemistry and pheromone activity, as reviewed by Mori (2002, 2004).

However, to our knowledge, this is the first example where unnatural stereoisomers are more active than the natural stereoisomer. Nevertheless, this observation will require further substantiation with studies of the interaction of

each stereoisomer with other pheromone components. For example, activity of the natural stereoisomer may be enhanced by other female pheromone components, whereas activity of the unnatural stereoisomers may not.

To resolve methodological uncertainties, it would be most informative to reexamine the behavior of the previously investigated cockroaches in Japan with the high-purity stereoisomers, to determine whether different populations of the German cockroach produce and respond to different stereoisomers of the contact sex pheromone.

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SEMIOCHEMISTRY OF THE GOLDENEYED LACEWING *Chrysopa oculata*: ATTRACTION OF MALES TO A MALE-PRODUCED PHEROMONE[1]

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Abstract—Gas chromatographic-electroantennographic detection (GC-EAD) experiments showed that antennae of males and females of the goldeneyed lacewing, *Chrysopa oculata* Say (*Co.* = *Chrysopa*), consistently responded to four compounds extracted from the abdominal cuticle of males: nonanal, nonanol, nonanoic acid, and (1*R**,2*S**,5*R**,8*R**)-iridodial. These compounds were not detected from abdominal cuticle of females. Thoracic extracts of both sexes contained antennal-stimulatory 1-tridecene and EAD-inactive skatole. *Chrysopa oculata* adults were most sensitive to (1*R*,2*S*,5*R*,8*R*)-iridodial standard at an EAD-response threshold between 0.1 and 1 pg, which was 10–100 times lower than thresholds for nonanal and nonanoic acid, and up to 10,000 times lower than thresholds for other compounds tested. A similar EAD response pattern was also found in another *Chrysopa* sp. (*Co. quadripunctata* Burmeister). In field-trapping experiments, (1*R*,2*S*,5*R*,8*R*)-iridodial was the only male-specific compound that attracted *Co. oculata* males. Males also were weakly attracted to (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (an aphid sex pheromone component), probably due to the 5% (1*R*,2*S*,5*R*,8*R*)-iridodial present in the synthetic sample as an impurity. A herbivore-induced plant volatile, methyl salicylate, increased attraction of males to (1*R*,2*S*,5*R*,8*R*)-iridodial, whereas 1-tridecene was antagonistic. No females were caught in the entire study. Scanning electron micrographs revealed numerous male-specific, elliptical epidermal glands on the 3rd–8th abdominal sternites of *Co. oculata*, which are

[1] Mention of commercial products does not constitute an endorsement by USDA.

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likely the pheromone glands. Another lacewing species, *Chrysoperla rufilabris* (Burmeister) (*Cl.* = *Chrysoperla*), did not produce male-specific volatiles or possess the type of gland presumed to produce pheromone in *Co. oculata* males, but (*Z*)-4-tridecene was identified as a major antennal-stimulatory compound from thoracic extracts of both sexes of *Cl. rufilabris*. Thus, (1*R*,2*S*,5*R*,8*R*)-iridodial (or its enantiomer) is now identified as a male-produced male aggregation pheromone for *Co. oculata*, the first pheromone identified for lacewings.

Key Words—Neuroptera, Chrysopidae, attractant, pheromone, synomone, kairomone, methyl salicylate, iridodial, tridecene, GC-EAD.

INTRODUCTION

Lacewings, especially green lacewings (Chrysopidae), are some of the most common predators of aphids and other soft-bodied insects (New, 1975; Tauber et al., 2000). Because of their commercial availability and resistance to insecticides, chrysopids are among the most commonly released predators for augmentative biological control (Ridgway and Murphy, 1984; Tulisalo, 1984; Aldrich, 1999) albeit with differing degrees of success (Rosenheim, 1998). Whereas green lacewings are increasingly being released for biocontrol, methods are still needed to retain the predators near augmentation sites and/or to attract wild populations to target areas (Baker et al., 2003).

Recent studies on semiochemicals involved in lacewing-related systems suggest that interspecific signals might improve biocontrol using lacewings. For example, various lacewings are attracted to host plant volatiles (Flint et al., 1979; Hagen, 1986; Zhu et al., 1999; Hooper et al., 2002), herbivore-induced plant volatiles (James, 2003), and sex pheromones of scale insects (Mendel et al., 2003), and aphids (Boo et al., 1998, 1999; 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*). However, the explanation that this attraction is a means to find aphid prey (Boo et al., 1998) has been questioned because only male lacewings are attracted, and aphids do not produce pheromone until late summer or autumn (Hooper et al., 2002). Hooper et al. (2002) suggested that aphid pheromone compounds, or analogous structures, may play a pheromonal role for lacewings, and that their similarity to aphid sex pheromones may be simply incidental.

Intraspecific chemical signals may have even more potential for managing lacewings, but pheromones of chrysopids have yet to be identified. Therefore, the present investigation was initiated to (1) search for pheromones of *Chrysopa oculata* Say (*Co.* = *Chrysopa*) and *Chrysoperla rufilabris* (Burmeister) (*Cl.* = *Chrysoperla*), the most common lacewings in the eastern United States, (2) compare the attraction of suspected pheromones and other known lacewing semiochemicals, and (3) test the potential antagonistic effect of allomone compounds on attraction.

METHODS AND MATERIALS

Adult Insects and Preparation of Extracts. Lacewings for chemical and GC-EAD analyses were collected in traps in late May 2003 during a preliminary field test (see below), and by sweep netting during June–August, 2003. Most *Co. oculata* adults were collected from an alfalfa field during daylight hours on the North Farm of the Beltsville Agricultural Research Center (BARC), Prince George's County, Maryland, whereas *Cl. rufilabris* adults were captured near a light in the backyard of a house in College Park, Maryland, between 21:30 and 23:30 hr. Adults of both species were dissected within 6–20 hr of capture for electrophysiological and chemical analyses. For extraction, *Co. oculata* adults were anesthetized with CO_2, eviscerated under tap water, the body parts were cut as follows, dried with tissue paper, and extracted individually in 50 μl of methyl tert-butyl ether: abdominal cuticle (segments 1–8), abdominal tip (last segment), and thorax. Thoracic and abdominal extracts of *Cl. rufilabris* were made in the same manner as for *Co. oculata*. All the extracts were kept at –20°C until GC-EAD/MS analyses. A few *Chrysopa quadripunctata* Burmeister males were collected from the sticky traps and used for GC-EAD analysis.

Gas Chromatography-Electroantennogram Detector (GC-EAD) Analysis. Lacewing extracts and chemical standards were analyzed in splitless mode using an HP 6890 GC equipped with a DB-WaxETR column (0.25 μm film thickness, 30 m × 0.25 mm ID; J & W Scientific, Folsom, CA), and a 1:1 effluent splitter that allowed simultaneous flame ionization detection (FID) 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 50°C/2 min, rising to 240°C at 10°C/min, then held for 10 min. The outlet for the EAD was held in a humidified air stream flowing at 0.5 m/sec over an antennal preparation. EAD recordings were made using silver wire-glass capillary electrodes filled with Beadle-Ephrussi Ringer (Zhang et al., 2000) on freshly cut antennae of both sexes. The antennal signals were stored and analyzed on a PC equipped with a serial IDAC interface box and the program EAD ver. 2.5 (Syntech, Hilversum, The Netherlands). In addition, GC-EAD dose-responses to a synthetic mixture containing five *Co. oculata*-produced compounds [1-tridecene, nonanal, nonanol, nonanoic acid, and (1*R*,2*S*,5*R*,8*R*)-iridodial]; three compounds associated with aphid prey: (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol and (4a*S*,7*S*,7a*R*)-nepetalactone (sex pheromone components), plus the nonpheromone compound, (4a*S*,7S,7a*S*)-nepetalactone; and one herbivore-induced plant volatile (methyl salicylate) were tested with both sexes of *Co. oculata*. Test compounds were dissolved in hexane (0.001, 0.01, 0.1, 1, 10, 100 ng/μl), and (*E*)-2-hexenyl butyrate (100 ng/μl) was used as positive control. For each GC-EAD run, ca. 2 μl of a test mixture plus 2 μl of the control were combined in the same syringe and then injected. Thus, after a 50:50 split at the end of the GC column, ca. 100 ng of (*E*)-2-hexenyl butyrate and 0.001 to 100 ng of each test compound (depending on the dosage) passed over the antennal

preparation. The EAD responses were then normalized as the percentage response to the control. The same synthetic mixture (100 ng/μl for each compound) was also tested on antennae of *Co. quadripunctata* (males only) and *Cl. rufilabris* (both sexes).

Chemical Standards (Figure 1). 1-Tridecene (97%), 1-nonanol (97%), and skatole (98%) were obtained from Aldrich Chemical (Milwaukee, WI), nonanal (99%) and (*E*)-2-hexenyl butyrate (98%) from Bedoukian Research (Danbury, CT), nonanoic acid (98%) from Emery Industries (Cincinnati, OH), and methyl salicylate (98%) from Fisher Scientific (Fair Lawn, NJ). (*Z*)-4-Tridecene was synthesized by condensing the ylide of *n*-butyltriphenyl phosphonium bromide with octanal following standard Wittig reaction procedure. The product was fractionally distilled to yield 98% 4-tridecenes (*Z*:*E* 93:7).

(4a*S*,7*S*,7a*R*)-Nepetalactone and (4a*S*,7*S*,7a*S*)-nepetalactone are often referred to as *Z*,*E*- and *E*,*Z*-nepetalactone, respectively, in the literature (e.g.,

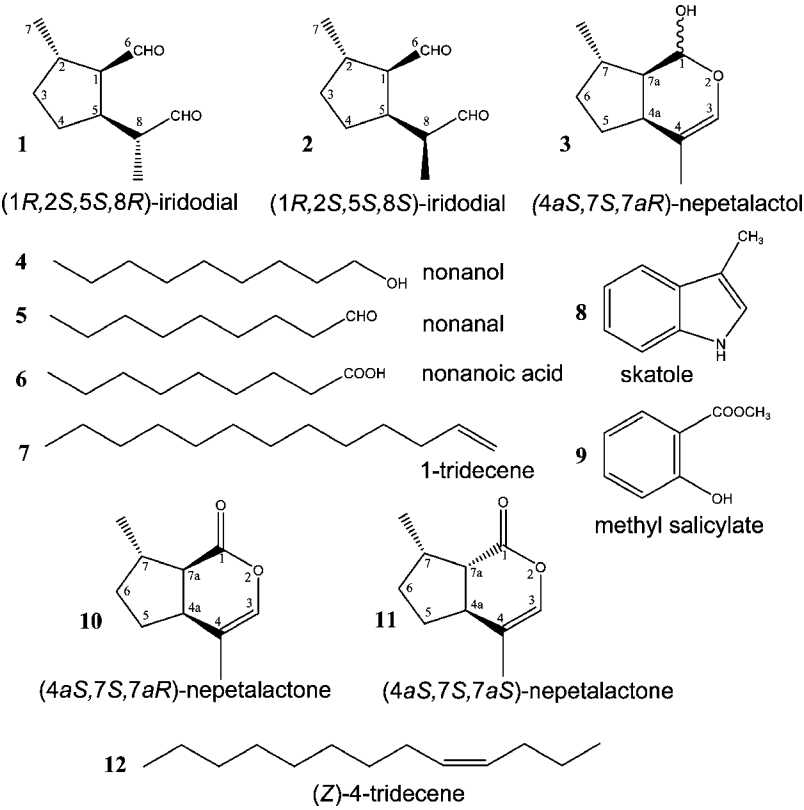

FIG. 1. Compounds identified and/or tested.

Hooper et al., 2002). (4a*S*,7*S*,7a*R*)-Nepetalactone (98%) and (4a*S*,7*S*,7a*S*)-nepetalactone (96%) used for the preliminary test in the spring of 2003 were isolated from commercial catnip oil (Health and Herbs, Philomath, OR) by silica gel flash chromatography using 5% ethyl acetate in hexane (Chauhan et al., 2004). (4a*S*,7*S*,7a*R*)-Nepetalactone (98%) used for later 2003 tests was prepared from commercial catnip oil by a pH-sensitive chemical separation technique (patent pending). (4a*S*,7*S*,7a*R*)-Nepetalactol [predominantly the 1*R*-isomer (Hooper et al., 2002), with ca. 5% impurity of iridodial isomers] was prepared by $NaBH_4$ reduction of (4a*S*,7*S*,7a*R*)-nepetalactone as previously described (Hooper et al., 2002). (1*R*,2*S*,5*R*,8*R*)-Iridodial [80%, with 20% (1*R*,2*S*,5*R*,8*S*)-iridodial as an impurity] was synthesized as described by Chauhan et al. (2004).

GC and GC-Mass Spectrometry (GC-MS) Analyses. Retention time (RT) comparisons, in addition to those provided by GC-EAD, and coinjections were performed in split mode using an HP 6890 GC equipped with a DB-5 column (0.25 μm film thickness, 30 m $\times$ 0.32 mm ID; J & W Scientific, Folsom, CA). Hydrogen was used as the carrier gas, programming from 50°C/2 min, to 250°C at 10°C/min, then held for 10 min. GC-MS was performed with an HP 6890 GC coupled to an HP 5973 mass selective detector using a DB-WaxETR column as above (except 60-m-long column), programmed at 50°C/2 min, rising to 230°C at 15°C/min, then held for 15 min. Male-produced compounds from *Co. oculata* were quantified by the internal standard method using (*E*)-2-hexenyl butyrate. Dimethyldisulfide (DMDS) derivatization (Buser et al., 1983) followed by GC-MS analysis was used to determine double bond positions.

Scanning Electron Microscopy. Live lacewings (males and females) of both species (*Co. oculata* and *Cl. rufilabris*) were anesthetized with CO_2, mounted on copper specimen holders (16 $\times$ 29 $\times$ 1.5 mm thick) with cryoadhesive, and immersed in liquid N_2. The frozen specimens were transferred to an Oxford CT1500 HF cryo preparation system, and examined using a low temperature scanning electron microscope (LTSEM; Hitachi S-4100) operated at 2.0 kV (see Erbe et al., 2003, for details). Micrographs were recorded on Polaroid Type 55 P/N film.

Field Trapping. A preliminary experiment was conducted from March 26 through June 5, 2003 to test potential repellent effects of nepetalactone isomers against the spined soldier bug (Heteroptera: Pentatomidae: *Podisus maculiventris* Say) and its parasitoids. In this test, clear plastic funnel traps baited with synthetic pheromone of the spined soldier bug were deployed as previously described (Aldrich et al., 1984) with or without nepetalactone isomers added. There were eight replicates per treatment plus unbaited control traps, and the nepetalactone treatments consisted of 15 mg of either (4a*S*,7*S*,7a*R*)-nepetalactone or (4a*S*,7*S*,7a*S*)-nepetalactone in100 μl CH_2Cl_2 applied to a separate rubber septum placed in the trap bottom.

Field-trapping experiments designed for *Co. oculata* were carried out in a meadow at BARC-East (experiment 1), and an alfalfa field on the BARC North Farm (experiments 2–3), from July through the beginning of September 2003, using Jackson delta sticky traps (Agrisense, Fresno, CA) baited with 5 mg of test compound(s) in 50 μl of heptane applied to grey rubber septa (5 mm sleeve-type; The West Co., Lititz, PA). Rubber septa were replaced twice per week. Traps were hung 0.8–1.0 m above ground on metal stakes ca. 8 m apart, with ca. 10 m between trap lines. For each experiment, 2–5 sets of traps (a set = one line of traps) were deployed with their initial trap positions being randomized within a set. The trap positions were then systematically rotated within a set after each replicate based on a procedure of Latin-square design (Byers, 1991) so that traps appeared at least once per location. To minimize positional effects, lacewing collections and trap rotations were carried out when $\geq$2 lacewings were caught in the best trap. Each replicate lasted 1–2 d, depending on flight activity. The sticky inserts were taken to the laboratory for recording lacewing species, gender, and numbers.

Experiment 1 (July 18 to August 1, 2003) was conducted with 5 sets of traps to determine behavioral activity of male-specific compounds that stimulated antennae in comparison with (4a*S*,7*S*,7a*R*)-nepetalactone and (4a*S*,7*S*,7a*R*)-nepetalactol. Experiment 2 (August 5 to September 2, 2003) consisted of two sets of traps to test potential activity of the four male-specific compounds, (1*R*,2*S*,5*R*,8*R*)-iridodial, nonanal, nonanol, and nonanoic acid, in a four-way factorial design, i.e., four individual components and all their possible binary, ternary, and quaternary blends (in the same ratio). Experiment 3 (August 21 to September 1, 2003) tested, in four sets of traps, the potential interaction between (1*R*,2*S*,5*R*,8*R*)-iridodial and methyl salicylate, and 1-tridecene. (4a*S*,7*S*,7a*R*)-Nepetalactone and a mixture of nonanal, nonanol, and nonanoic acid (at 1:1:1; on one dispenser) were also included for comparison.

Statistical Analysis. Because of heterogeneity of variances among treatments, trap catch data (number of lacewings caught/trap/replicate) were analyzed using the nonparametric Kruskal-Wallis ANOVA on rank test, followed by the Student-Newman-Keuls all pair-wise comparison to separate means (Zar, 1984).

RESULTS

GC-EAD and Chemical Identifications. In general, antennal preparations of all three lacewing species tested remained active for at least 2 hr, which allowed time for 3–4 GC-EAD runs per preparation. The response of *Co. oculata* antennae toward synthetic (4a*S*,7*S*,7a*R*)-nepetalactol (**3**) is shown in Figure 2A; the antennae of both males and females (not shown) were much more responsive to two impurities in this synthetic standard (**1** and **2**; RT = 15.15 and 15.40 min, respectively) than to nepetalactol (**3**). The GC trace of the *Co. oculata* 1st–8th abdominal

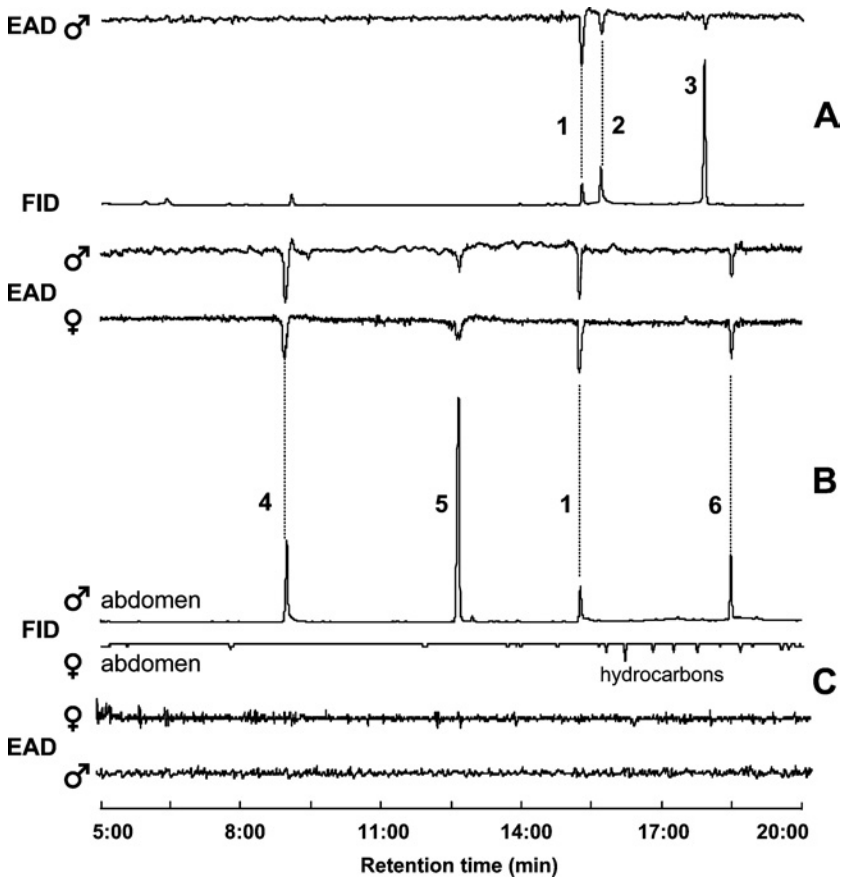

FIG. 2. GC-EAD responses of *Chrysopa oculata* antennae to (A) synthetic (4a*S*,7*S*,7a*R*)-nepetalactol (**3**, see Figure 1), and to cuticular extracts of the 1st–8th abdominal segments of conspecific males (B) and females (C).

extract from males revealed four main peaks (Figure 2B), none of which was observed in the corresponding extract from conspecific females (Figure 2C). The major component of the *Co. oculata* male abdominal extract (**5**) elicited the weakest EAD response of the four major components in this extract. The other three main components elicited strong responses from the antennae of both sexes, with the response to **1** consistently being strongest and the RT of **1** matching that of the first EAD-active impurity in the synthetic (4a*S*,7*S*,7a*R*)-nepetalactol standard (Figure 2B). In contrast, no EAD-active compounds were detected from extracts of the 1st–8th abdominal cuticle of *Co. oculata* females (Figure 2C) or abdominal tip extracts (not shown).

GC-MS analyses revealed that the earlier eluting EAD-active impurity in the nepetalactol standard and the corresponding *Co. oculata* male-specific component (Figure 2A and B, respectively) exhibited identical MS data: *m/z* (%) 67 (65), 81 (100), 109 (35), 135 (18), 150 (8), 153 (4), 168 (3, M^+). Nepetalactol is a monoterpene enol-lactol that exists in equilibrium with iridodials depending on physiochemical conditions (El-Naggar and Beal, 1980). The mass spectra of these natural products matched the published spectra for iridodial (Cavill et al., 1976). Because iridodial standards **1** and **2** were derived from (*Z*,*E*)-nepetalactone **10**, the absolute configuration remains intact for the (7a)-, (7)-, and (4a)-positions of origin (Dawson et al., 1996). The absolute configuration of the newly generated chiral center at C-8 in iridodials **1** and **2** was established by nuclear magnetic resonance spectroscopy (Chauhan et al., 2004). In iridodial **1**, the coupling constant between H5 and H8 ($J = 13.2$ Hz) indicated a *threo* or *trans* configuration (8*R*), whereas in iridodial **2**, $J = 11.3$ Hz between H5 and H8 confirmed an *erythro* or *cis* configuration (8*S*). The relative stereochemistry of lacewing-derived iridodial was identified by coinjection of natural extract with synthetic standards of (1*R*,2*S*,5*R*,8*R*)- and (1*R*,2*S*,5*R*,8*S*)-iridodial. The lacewing-derived material coeluted with (1*R*,2*S*,5*R*,8*R*)- iridodial (RT = 13.30 min), confirming the structure of the *Co. oculata* male-specific product as (1*R*,2*S*,5*R*,8*R*)-iridodial (**1**) or the enantiomer, (1*S*,2*R*,5*S*,8*S*)-iridodial. Male-specific compounds **4**, **5**, and **6** from *Co. oculata* were identified as nonanal, nonanol, and nonanoic acid, respectively, by matching GC and GC-MS data to that of the known standards. The *Co. oculata* male-specific compounds were quantitated as follows (mean μg/male $\pm$ SE; $N = 9$): nonanal (0.91 ± 0.3), nonanol (2.2 ± 0.5), (1R^*,2S^*,5R^*,8R^*)-iridodial (0.26 ± 0.1), and nonanoic acid (0.35 ± 0.1). The coefficients of variation (CV = SD $\times$ 100/mean) in relative amounts (= proportions) of the individual male-specific components were 37% (**4**), 20% (**5**), 112% (**1**), and 56% (**6**).

GC-EAD analyses of thoracic extracts of *Co. oculata* indicated the presence of two main compounds (**7** and **8**), but only the former component was EAD-active (Figure 3). These compounds were identified as 1-tridecene (**7**) and skatole (**8**) by GC and GC-MS comparisons with standards, confirming their earlier identification from this species (Blum et al., 1973).

GC-EAD analyses with *Co. oculata* antennae using synthetic mixtures showed that nonanal, nonanoic acid, and (1*R*,2*S*,5*R*,8*R*)-iridodial elicited much higher EAD responses than did nonanol (**5**), 1-tridecene (**7**), (4a*S*,7*S*,7a*R*)-nepetalactone (**10**), (4a*S*,7*S*,7a*S*)-nepetalactone (**11**), 4a*S*,7*S*,7a*R*-nepetalactol (**3**), or methyl salicylate (**9**) (Figure 4). There were no significant differences in antennal responses between males and females in these tests. GC-EAD dose–response analyses (doses ranging from 1 pg up to 100 ng for each tested compound) showed a significant dose-effect for all the compounds tested. The average EAD response for 100 ng of (*E*)-2-hexenyl butyrate, the active control, was ca. 0.2 mV ($N = 53$). Antennae of both sexes of *Co. oculata* were most

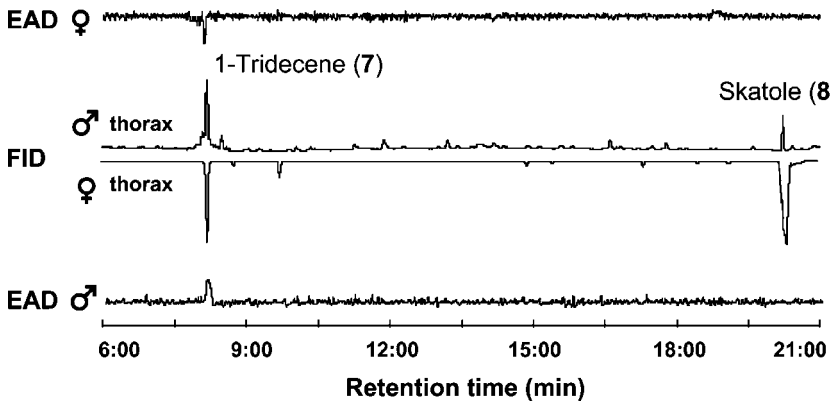

FIG. 3. GC-EAD responses of *Chrysopa oculata* to thoracic extracts of goldeneyed lacewing adult males and females.

sensitive to (1*R*,2*S*,5*R*,8*R*)-iridodial with its EAD-response threshold being between 0.1 and 1 pg (or lower), which is 10–100 times lower than thresholds for nonanal and nonanoic acid (Figure 4A), and ca. 1000–10,000 times lower than others (Figure 4A and B). Male antennae of another *Chrysopa* species, *Co. quadripunctata* (females were not available for test), showed a similar EAD response pattern to the synthetic mixtures as that seen for *Co. oculata* (Figure 5B).

Antennae of *Chrysoperla rufilabris* showed similar EAD responses to most of the compounds in the synthetic mixture (only 100-ng dose tested), but were unresponsive to (1*R*,2*S*,5*R*,8*R*)-iridodial (**1**) and its (8*S*)-stereoisomer (**2**) (Figure 5A). Volatile compounds were not detected in abdominal cuticular extracts of either sex by GC or EAD in *Cl. rufilabris* (not shown). Thoracic extracts of *Cl. rufilabris* indicated the presence of one main component (**12**), which elicited EAD-responses (Figure 6). GC-MS analyses of the thoracic extracts showed that the spectrum of the main compound contained ions characteristic of a tridecene (M^+ at m/z 182), the 4–5 double bond position was determined by DMDS derivation followed by GC-MS analysis, and GC coinjection with standards established the structure of this natural product as (*Z*)-4-tridecene. The mass spectrum of the minor component eluting within 1 min after **12** indicated a molecular weight of 180, suggesting a C_{13}-diene structure. Further characterization of this compound was not pursued because this compound did not stimulate antennae in GC-EAD analyses.

Scanning Electron Microscopy. Elliptical epidermal glands (ca. 12 × 7.5 μm with a central slit) occur in great numbers on the 3rd–8th abdominal sternites of *Co. oculata* males (Figure 7A). These glands do not occur on the 1st, 2nd, or

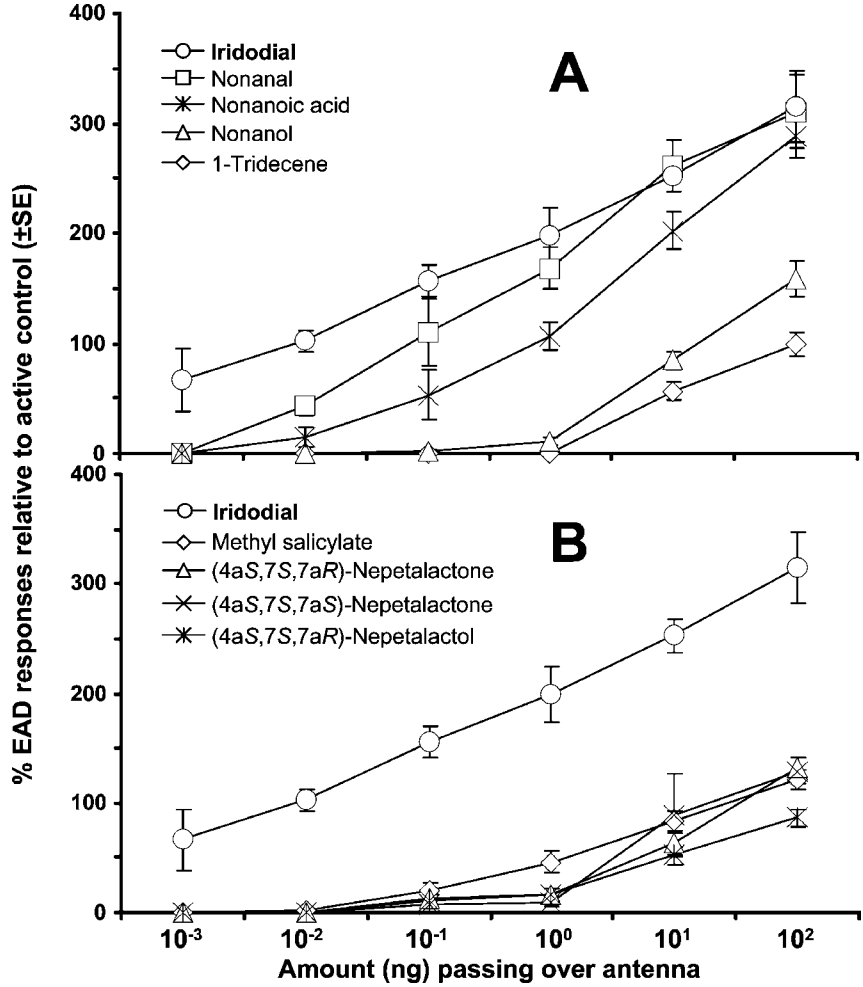

FIG. 4. GC-EAD dose-responses of *Chrysopa oculata* to synthetic mixtures ranging from 1 pg to 100 ng per test compound. (A) Comparison of (1*R*,2*S*,5*R*,8*R*)-iridodial with other *Co. oculata*- produced compounds; (B) comparison of (1*R*,2*S*,5*R*,8*R*)-iridodial with prey- or plant-produced compounds. For each dosage and compound, 4–17 GC-EAD runs were conducted. Pooled data (both male and female antennae) were used due to the lack of differences in EAD responses between sexes. The responses were normalized as the response (%) relative to an active control (100 ng of (*E*)-2-hexenyl butyrate; Mean EAD = 0.2 mV).

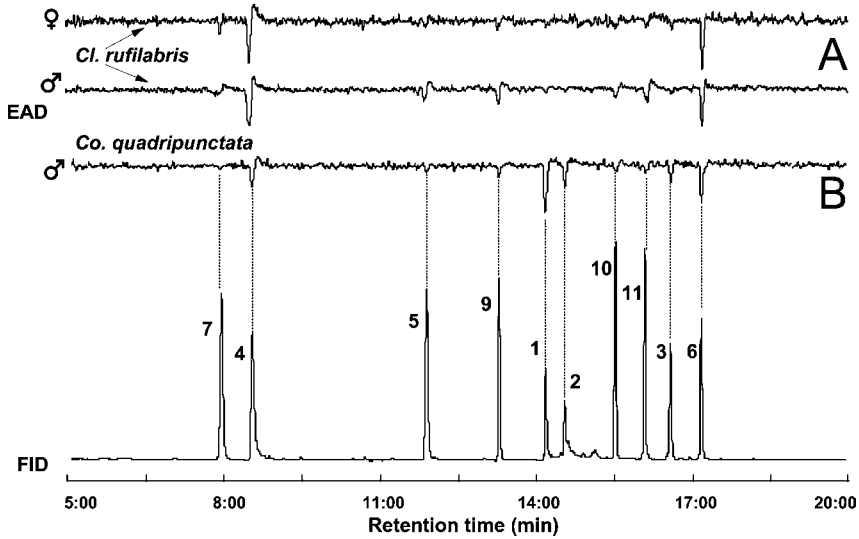

FIG. 5. GC-EAD responses of *Chrysoperla rufilabris* male and female (A), and *Chrysopa quadripunctata* male (B) to a synthetic standards (100 ng/μl each; numbers refer to structures in Figure 1).

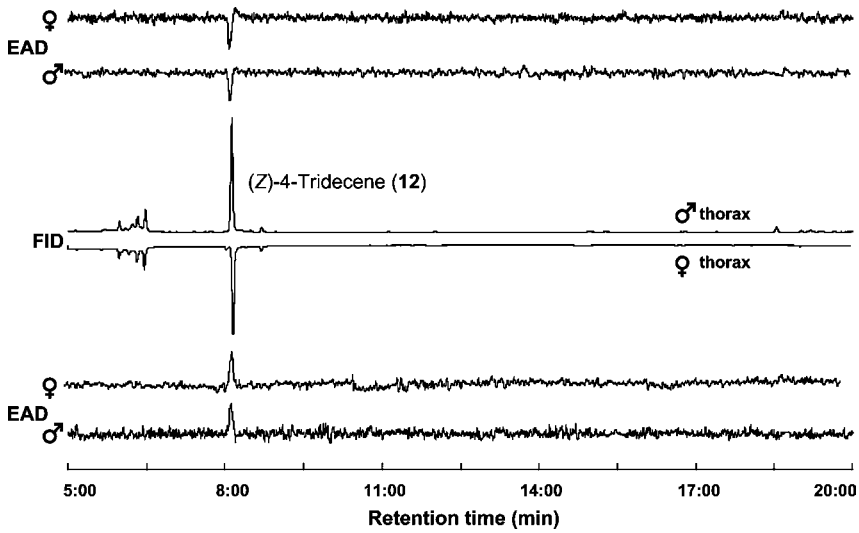

FIG. 6. GC-EAD responses of *Chrysoperla rufilabris* to thoracic extracts of conspecific males and females.

9th abdominal sternites of males, and are totally absent in *Co. oculata* females (Figure 7B). Sternites 3–8 were estimated to have about 800, 2100, 2500, 2500, 2300, and 1500 of these epidermal glands, respectively. The male-specific gland system evident in *Co. oculata* is totally absent in both males and females of *Cl. rufilabris* (Figure 7C and D, respectively). No SEMs of *Co. quadripunctata* adults were prepared.

Preliminary Field Trapping Experiment. A total of eight *Co. oculata* males were captured in clear plastic traps baited with spined soldier bug pheromone plus (4a*S*,7*S*,7a*R*)-nepetalactone (*Z*,*E*-nepetalactone) from May 30 through June 5, 2003. No *Co. oculata* females were captured, nor were any *Co. oculata* adults caught in unbaited control traps or traps baited with other treatments.

Field Trapping Experiments for Co. oculata. In experiment 1, a total of 57 *Co. oculata* males were caught in five sets of traps. Traps baited with (1*R*,2*S*,5*R*,8*R*)-iridodial or (4a*S*,7*S*,7a*R*)-nepetalactol [with 5% of (1*R*,2*S*,5*R*,8*R*)-iridodial as impurity] caught significantly more males than did blank control traps, whereas the mixture of the three male-produced C_9-compounds (**4–6**) and (4a*S*,7*S*,7a*R*)-nepetalactone (**10**) alone were each inactive (Figure 8). (1*R*,2*S*,5*R*,8*R*)-Iridodial was 4 times more attractive than (4a*S*,7*S*,7a*R*)-nepetalactol. No females were captured in the entire test.

In experiment 2, 690 *Co. oculata* males were caught. The individual C_9-compounds (**4–6**) did not attract any lacewings, and the binary and ternary combinations of these compounds were also inactive (Figure 9). Traps baited with (1*R*,2*S*,5*R*,8*R*)-iridodial alone or (1*R*,2*S*,5*R*,8*R*)-iridodial plus the three C_9-compounds in all possible combinations caught significant numbers of *Co. oculata* males. However, addition of the C_9-compounds to (1*R*,2*S*,5*R*,8*R*)-iridodial significantly reduced the trap catches (Figure 9). No females were caught in any traps in this experiment, although four eggs were found on the outside of one trap baited with (1*R*,2*S*,5*R*,8*R*)-iridodial, and 2–3 females were observed landing on the plants close to the (1*R*,2*S*,5*R*,8*R*)-iridodial baited traps. In addition, four males of *Co. quadripunctata* were captured in traps having lures containing (1*R*,2*S*,5*R*,8*R*)-iridodial.

In experiment 3, 454 males of *Co. oculata* were captured. (1*R*,2*S*,5*R*,8*R*)-Iridodial alone was again attractive (Figure 10). Traps baited with the C_9-blend (loaded on one dispenser), 1-tridecene, or methyl salicylate caught no lacewings, whereas three lacewings were caught in traps baited with (4a*S*,7*S*,7a*R*)-nepetalactone. Both 1-tridecene and the C_9-blend showed significant inhibitory effects on attraction of male lacewings to (1*R*,2*S*,5*R*,8*R*)-iridodial, with inhibitory effect of 1-tridecene being stronger than that of the C_9-blend. Combining (1*R*,2*S*,5*R*,8*R*)-iridodial and methyl salicylate resulted in a significant increase in the number of *Co. oculata* males that were caught relative to traps baited with (1*R*,2*S*,5*R*,8*R*)-iridodial alone (Figure 10). Again, no females were caught in the entire experiment.

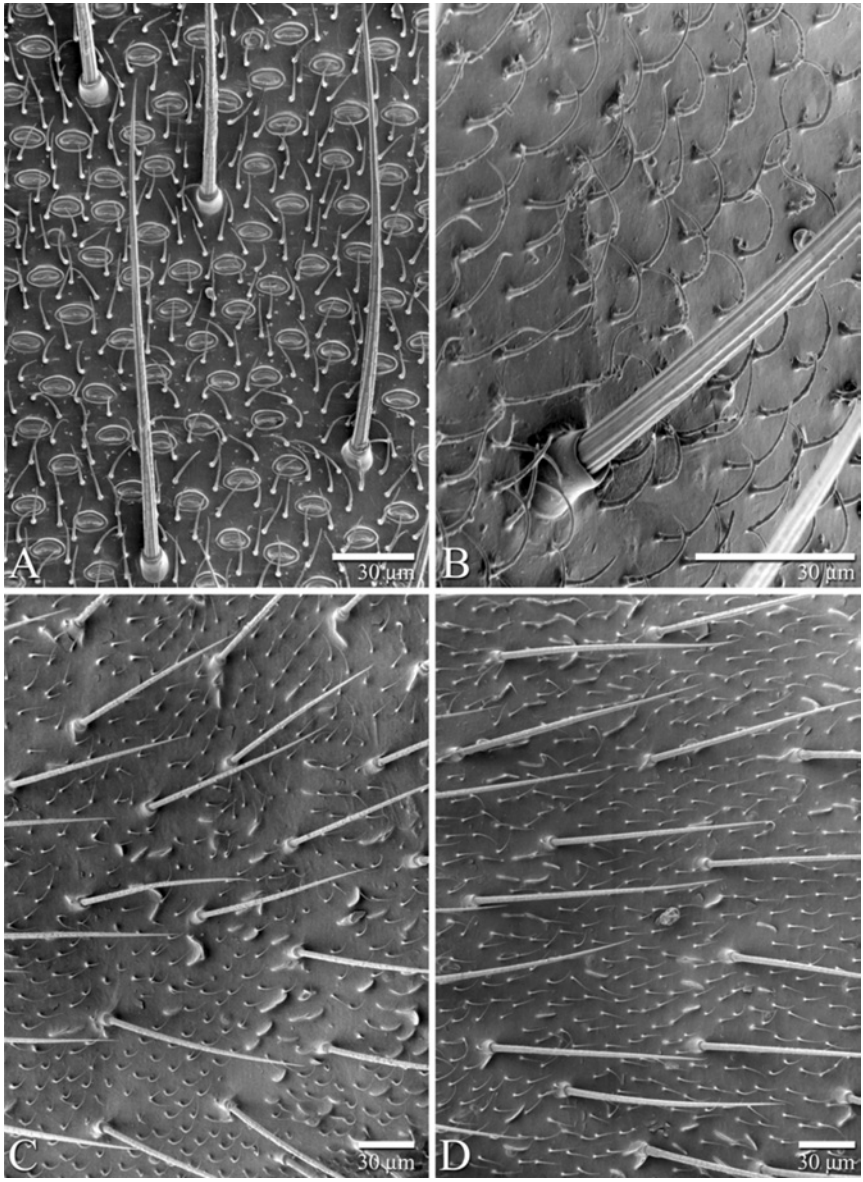

FIG. 7. SEM images of the 5th abdominal sternite of *Chrysopa oculata* and *Chrysoperla rufilabris*: *Chrysopa oculata* male (A) and female (B); *Chrysoperla rufilabris* male (C) and female (D).

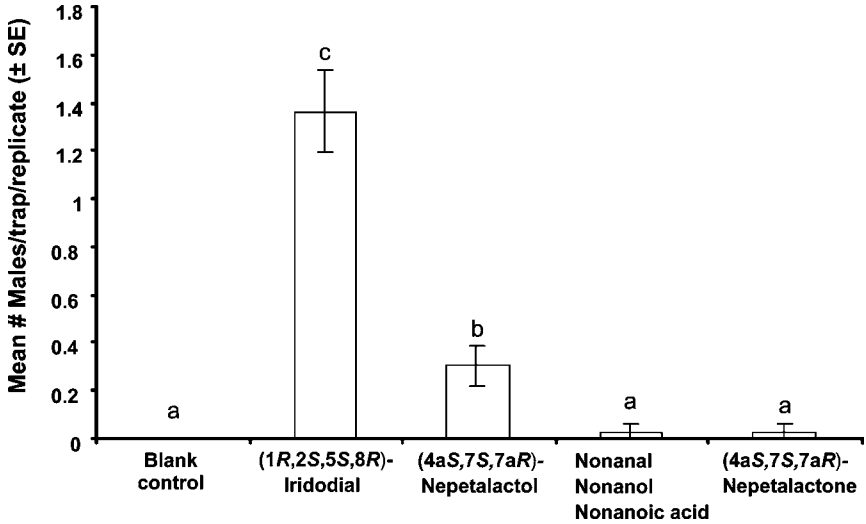

FIG. 8. Captures of male *Chrysopa oculata* in traps baited with (1*R*,2*S*,5*R*,8*R*)-iridodial, the C_9-blend, (4a*S*,7*S*,7a*R*)-nepetalactone, or (4a*S*,7*S*,7a*R*)-nepetalactol ($N = 33$, $\Sigma = 57$). Means followed by the same letter are not significantly different ($P > 0.05$), Kruskal-Wallis ANOVA on ranks, followed by Student-Newman-Kuels all pairwise comparison.

(1*R*,2*S*,5*R*,8*R*)-Iridodial, the C_9-blend, and other compounds eliciting responses from *Cl. rufilabris* antennae [(4a*S*,7*S*,7a*R*)-nepetalactone, (4a*S*,7*S*,7a*R*)-nepetalactol, and methyl salicylate] were also tested during August and September 2003, in a residential area where *Cl. rufilabris* was abundant. Over a 2-wk period using two sets of traps, 1 male and 1 female *Cl. rufilabris* were caught in the traps baited with methyl salicylate, whereas none were caught in traps baited with (1*R*,2*S*,5*R*,8*R*)-iridodial or the other compounds.

DISCUSSION

Our results with the goldeneyed lacewing, *Chrysopa oculata*, clearly indicate that (1R^*,2S^*,5R^*,8R^*)-iridodial is a male-produced pheromone that attracts conspecific males. The pheromone is probably produced in elliptical glands abundantly distributed on the 3rd–8th abdominal sternites of males. Another lacewing common in our region, *Chrysoperla rufilabris*, completely lacked the pheromone system found in the goldeneyed lacewing.

This is the first pheromone identified for lacewings, a discovery supporting speculation by Hooper et al. (2002) that attraction of male lacewings to nepetalactone and nepetalactol components of aphid sex pheromones, might be

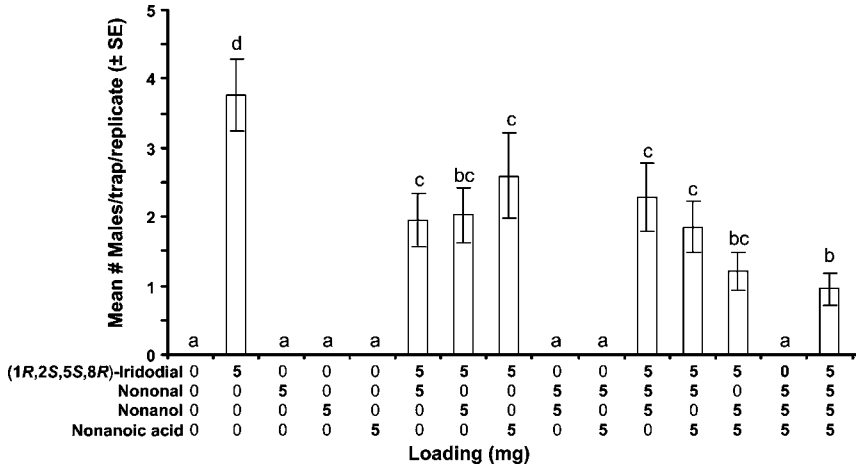

FIG. 9. Captures of male *Chrysopa oculata* in traps baited with four individual male-specific compounds, (1*R*,2*S*,5*R*,8*R*)-iridodial, nonanal, nonanol, and nonanoic acid, and all possible binary, ternary, and quaternary combinations ($N = 39$, $\Sigma = 690$). Means followed by the same letter are not significantly different ($P > 0.05$), Kruskal-Wallis ANOVA on ranks, followed by Student-Newman-Kuels all pairwise comparison.

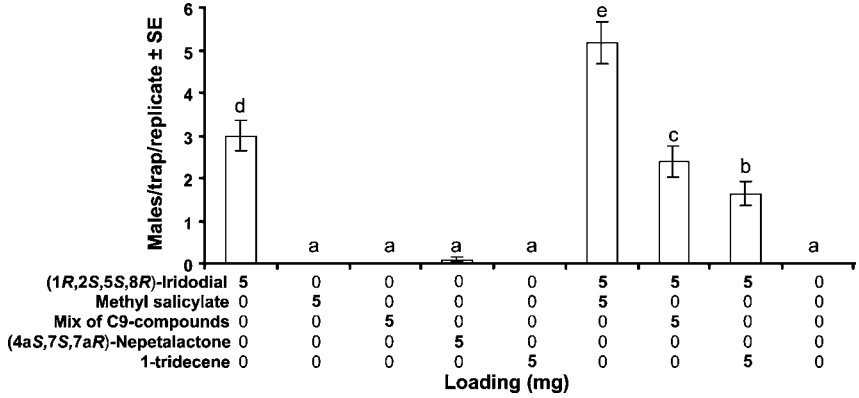

FIG. 10. Captures of male *Chrysopa oculata* in traps baited with (1*R*,2*S*,5*R*,8*R*)-iridodial alone, the C_9-blend, (4a*S*,7*S*,7a*R*)-nepetalactone, or methyl salicylate, and with some combinations of these compounds ($N = 36$, $\Sigma = 454$). Means followed by the same letter are not significantly different ($P > 0.05$), Kruskal-Wallis ANOVA on ranks, followed by Student-Newman-Kuels all pairwise comparison.

incidental to the fact that they prey upon aphids. Isomers of iridodial are unavoidable impurities in nepetalactol (Chauhan et al., 2004), the aphid pheromone component most attractive to *Chrysopa* spp. (Boo et al., 1999). In our preliminary testing, we found that *Co. oculata* males [(probably matured from overwintered prepupae (Canard and Principi, 1984)] were attracted in late spring to traps containing 15 mg of (4a*S*,7*S*,7a*R*)-nepetalactone (*Z*,*E*-nepetalactone) isolated chromatographically from catnip oil, similar to an earlier report (Boo et al., 1998). However, the tests conducted later in the season using a 5-mg dose of (4a*S*,7*S*,7a*R*)-nepetalactone isolated chemically from catnip oil by a different procedure attracted fewer lacewings than did traps baited with 5 mg of (4a*S*,7*S*,7a*R*)-nepetalactol.

Iridodials have been identified from many other natural sources such as ants (Attygalle and Morgan, 1984; Nascimento et al., 1998), especially the *Iridomyrmex* spp. (e.g., Cox et al., 1989), as well as rove beetles (Staphylinidae) (Huth and Dettner, 1990), and a stick insect (Phasmatodea: Phasmatidae) (Smith et al., 1979). In all these cases, iridodials serve as defensive compounds (Attygalle and Morgan, 1984). Iridodial is even claimed to be useful in the prevention of human cancer (Anonymous, 2002), although we were unable to detect any iridodials in the commercial product (Iridodial, Global Healing Center, Houston, TX) (unpublished data).

Whereas ours is the first report of a pheromone for lacewings, chrysopids have long been known to communicate acoustically (Henry, 1982). Chrysopids produce low frequency, substrate-borne vibrations that guide the sexes to find one another on a plant (Henry, 1982). Comparative acoustic studies of *Chrysoperla* vs. *Chrysopa* species indicate that *Chrysoperla* spp. [*Cl. rufilabris*, *Cl. carnea* (Stephens) and *Cl. downesi* (Smith)] are more dependent on acoustic signals for mating success than are *Chrysopa* spp. (*Co. oculata* and *Co. 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 and, to a lesser extent than *Chrysoperla*, with acoustic signals. Adults of *Chrysopa* (*sensu stricto*) are predacious, whereas *Chrysoperla* adults are phytophagous (Principi and Canard, 1984), suggesting that predation in the adult stage somehow favors chemical communication or selects against communication by substrate vibration. The work of Castellanos (2003; Castellanos and Barbosa, 2004) provides an example of how silence is favored in predatory insects: the spined soldier bug, *Podisus maculiventris* Say (Heteroptera: Pentatomidae), is a more successful predator of certain caterpillars than are *Polistes* paper wasps, in large part because the bug is not as noisy while hunting as are the wasps.

Surprisingly, no females of *Co. oculata* were caught during any of our experiments, even though antennae from females were as sensitive to (1*R*,2*S*,5*R*,8*R*)-iridodial and other male-produced compounds as were antennae

of males. However, four lacewing eggs were laid on one trap baited with (1*R*,2*S*, 5*R*,8*R*)-iridodial, and three females were observed on plants nearby (1*R*,2*S*, 5*R*,8*R*)-iridodial baited traps. Therefore, it is possible that female lacewings are also attracted to (1*R*,2*S*,5*R*,8*R*)-iridodial, but do not approach close enough to be trapped. Once a female is in the vicinity of a pheromone-calling male, it is conceivable that she produces substrate vibrations that cause the male to approach her. Indeed, in phytophagous bugs such as the southern green stink bug, *Nezara viridula* (L.) (Pentatomidae), long-range attraction to male-produced pheromones probably brings both sexes together on the same plant (Aldrich, 1995), and then substrate-borne vibrations produced by the female stimulate males to walk to the female (Çokl et al., 1999; Çokl and Virant-Doberley, 2003).

In addition to (1*R**,2*S**,5*R**,8*R**)-iridodial, the three antennal-stimulatory active C_9-compounds (nonanal, nonanol, and nonanoic acid) were also found in abdominal cuticular extracts of males. These C_9-compounds alone or in blends were unattractive to *Co. oculata*, and reduced attraction of the males to (1*R*,2*S*,5*R*,8*R*)-iridodial. Individual variation in the relative amounts of (1*R**,2*S**,5*R**,8*R**)-iridodial (coefficient of variation = 112%) is much greater than that for the C_9-compounds (CV = 20–56%). Such a large individual variation for iridodial might indicate that the sampled *Co. oculata* males were physiologically different, and that the C_9-compounds may play a role independent from that of iridodial. As one might expect for insect pheromones, the EAD-response threshold of *Co. oculata* to (1*R*,2*S*,5*R*,8*R*)-iridodial (0.1–1 pg) was 10–100 times lower than that for nonanal and nonanoic acid, and 1000–10,000 times lower than that observed for nonanol, 1-tridecene, and other compounds tested. *Chrysopa quadripunctata* also showed an EAD-response pattern similar to that of *Co. oculata*, but too few insects were available to pursue pheromone identification. *Chrysoperla rufilabris* showed no EAD response to (1*R*,2*S*,5*R*,8*R*)-iridodial, which correlates with lack of behavioral responses to this compound by *Cl. rufilabris* adults. Interestingly, however, *Cl. rufilabris* antennae were about as sensitive to the C_9-compounds as were *Co. oculata* antennae, which supports independent roles for iridodial and the C_9-compounds. Our analysis of the abdominal tip extract failed to detect EAD-active compounds, even though *Co. oculata* males possess eversible vesicles near the tips of their abdomens that are suspected to be pheromone glands (Hwang and Bickley, 1961).

Elliptical epidermal glands occur on the 3rd–8th abdominal segments in several European *Chrysopa* species (Principi, 1949, 1954a,b). In *Co. septempunctata* Wesmael (Principi, 1949) and *Co. viridana* Schneider (Principi, 1954a), the glands are distributed on both tergites and sternites, whereas in *Co. perla* (L.), these glands exist only on the sternites (Principi, 1954b), a distribution as in *Co. oculata*. In other lacewing species, such as *Pseudomallada flavifrons* (Brauer) and *P. ventralis* (Curtis), similar integumental structures occur on the male thoracic

tergites, whereas in *Cl. carnea*, *Nineta flava* (Scopoli), and *N. vittata* (Wesmael) (Principi, 1954b), as well as in *Cl. rufilabris* (this paper), elliptical glands are absent. On the basis of our SEM observations of *Co. oculata*, we estimate that a male has almost 12,000 elliptical glands (ca. 2,000/mm^2), a glandular abundance equivalent to that of *Co. septempunctata* (Principi, 1949). The fact that the distribution of elliptical glands in *Co. oculata* males coincides with extraction of the pheromone from the 3rd–8th abdominal sternites strongly implicates these glands as the source of the pheromone. We suspect that *Co. quadripunctata*, and other chrysopid species with epidermal glands as found in *Co. oculata* males, possess pheromone systems similar to that of the goldeneyed lacewing. In fact, the attraction of males of various chrysopid species to dihydronepetalactols (neomatatabiol and isoneomatatabiol) from the Japanese plant, *Actinidia polygama* Miq. (Actinidiaceae), may be due to the production of these or related compounds from male chrysopids themselves (Hyeon et al., 1968; Sakan et al., 1970; Hooper et al., 2002).

Chemical defense in the goldeneyed lacewing (Blum et al., 1973) and other chrysopids (Zhu et al., 2000) is thought to be accomplished by secretion from a pair of thoracic glands. Our analyses of *Co. oculata* thoracic extracts confirmed the results of Blum et al. (1973) that 1-tridecene and skatole are produced by both sexes. Surprisingly, skatole, a compound strongly organoleptic to humans and other animals, elicited no EAD response from *Co. oculata*, whereas 1-tridecene elicited a strong response from antennae of both sexes of this species. In our study, addition of 1-tridecene to (1*R*,2*S*,5*R*,8*R*)-iridodial-baited traps significantly reduced the numbers of *Co. oculata* males captured. This antagonistic effect indicates that 1-tridecene might also function as an alarm or antiaggregation pheromone. In contrast to *Co. oculata*, no skatole was detected from thoracic extracts of *Cl. rufilabris*, but a significant EAD response was elicited from antennae of both sexes to the major component (common in both males and females) identified as (*Z*)-4-tridecene. Similarly, in *Cl. carnea*, (*Z*)-4-tridecene was identified as the main thoracic gland component, and stimulated antennae, but skatole was absent (Zhu et al., 2000).

Whereas attraction of chrysopids to nepetalactone, nepetalactol, and dihydronepetalactols may not be directly related to the occurrence of these compounds in plants, at least some herbivore-induced plant volatiles appear to be ecologically relevant signals guiding lacewings to prey-infested plants (Han and Chen, 2002; James, 2003). Methyl salicylate, a volatile induced by phloem-feeding whiteflies and aphids (Walling, 2000), significantly attracted males and females of *Co. nigricornis* in the field (James, 2003). Our tests of methyl salicylate at a dose of 5 mg/lure, a much lower dose than that used by James (2003), failed to attract *Co. oculata*. However, methyl salicylate significantly increased attraction of *Co. oculata* males to (1*R*,2*S*,5*R*,8*R*)-iridodial. This synergism between iridodial (a pheromone) and methyl salicylate (a synomone in this context) supports the

concept that coincidence of chemical signals from different trophic levels together constitutes a more powerful attractant blend than a monotrophic signal (Zhang and Schlyter, 2003). (1*R*,2*S*,5*R*,8*R*)-Iridodial alone or its combination with herbivore-induced plant volatiles (e.g. methyl salicylate) and/or prey-produced kairomones may be of practical utility in manipulating natural or artificially augmented populations of lacewings for enhanced biological control.

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COUMARIN DIFFERENTIALLY AFFECTS THE MORPHOLOGY OF DIFFERENT ROOT TYPES OF MAIZE SEEDLINGS

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Abstract—The effects of coumarin on the length, diameter, and branching density of different root types in maize seedlings (*Zea mays* L. cv. Cecilia) were investigated. The maize root system represents a useful model for morphological studies, as it consists of radicle, seminal, and nodal roots whose origin and development are quite different. Maize seedlings were grown in a hydroponic culture for 6 days, and then coumarin (at concentrations of 0, 25, 100, and 400 μM) was added to the nutrient solution. Coumarin inhibited root length, but effects differed depending on the root type. $C_{1/2}$ values, representing the coumarin concentration causing a 50% inhibition of the root length, were calculated by nonlinear regression. Six, 1, and 0.25 mM coumarin were sufficient to reduce the radicle, seminal, and nodal root lengths by 50%, respectively. At the highest coumarin concentration, the subapical root zone showed swelling. The degree recorded by average diameter was higher in nodal roots than in seminal and radicle roots. Furthermore, coumarin decreased the number of lateral roots and branching density more in the seminal than in the radicle roots. These results suggest the following order of sensitivity to coumarin: nodal > seminal > radicle roots. The observed spatial effects of coumarin could be ecologically significant, since taprooted species could benefit at the disadvantage of fibrous-rooted species and could modify community composition.

Key Words—Coumarin, maize, root morphology, root types.

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INTRODUCTION

An important aspect of allelopathy research is understanding the ecological role of allelochemicals, which are released into the environment from living plants or decomposing plant residues (Rice, 1984), and involved in plant–plant interactions in both natural and agricultural ecosystems (Einhellig, 1995).

Coumarins, lactones of *o*-hydroxycinnamic acid, constitute a large class of allelochemicals. Coumarin (1,2-benzopyrone) represents the simplest compound in this class. It either promotes or inhibits plant growth, and the response is species-specific and concentration-dependent (Murray et al., 1982).Several physiological processes are affected by coumarin: it decreases respiration and photosynthesis by inhibiting electron transport (Moreland and Novitzky, 1987); it promotes cell differentiation in *Petunia* leaf explants (Abenavoli et al., 2001b); and it interferes with nitrate uptake in wheat seedlings (Abenavoli et al., 2001a) and nitrogen metabolism in carrot cell suspension culture (Abenavoli et al., 2003). Several investigations have indicated that the main effect of coumarin is inhibition of root growth (Goodwin and Taves, 1950; Svensson, 1971; Aliotta et al., 1993; Kupidlowska et al., 1994; Abenavoli et al., 2001a). However, studies targeting the effects of coumarin (Svensson, 1971; Kupidlowska et al., 1994; Abenavoli et al., 2001a) and/or other allelochemicals on roots, have focused on single primary roots of different plant species (Jankay and Muller, 1976; Aliotta et al., 1993; Abrahim et al., 2000). Furthermore, the morphological changes in the seminal and/or lateral roots were observed only visually or photographically without providing quantitative data (Vaughan and Ord, 1991; Baziramakenga et al., 1994).

Root systems are composed of several root types, characterized by different anatomical, morphological, and physiological features (Waisel and Eshel, 2002). These are genetically determined, but respond differently to soil environmental stresses (Zobel, 1991, 1995). Thus, for a better understanding of the effects of allelochemicals on plant roots, the morphological variability within a root system needs to be considered.

In the present work, maize seedlings were used as a model system to study the effects of coumarin on roots, since maize develops a complex root system, characterized by radicle, seminal, and nodal roots, differing in origin, development, physiology, and anatomy (McCully, 1999). The morphological changes of different root types in response to coumarin were investigated at different concentrations by digital technology. Root length, diameter, and branching density were recorded as morphological parameters, because they play a fundamental role in the acquisition of soil resources (Ryser, 1998). The information obtained ought to advance our understanding of how plants adapt to soil environmental stresses, such as allelochemicals.

METHODS AND MATERIALS

Plant Material and Growth Conditions. Seeds of *Zea mays* L. cv. Cecilia were washed in tap water, surface sterilized for 20 min in 20% (v/v) sodium hypoclorite solution, and rinsed in distilled water (3×). Seeds were pregerminated at 24°C in darkness on filter paper moistened with 0.5 mM $CaSO_4$ for 36 hr. Ten seedlings were transferred into a growing unit containing 5 l of aerated one-fourth-strength Hoagland solution (Hoagland and Arnon, 1950). The pH was adjusted to 6.0 with 0.1 N KOH and the growing units were placed into a growth chamber at 24°C with a 14-hr photoperiod (300 μmol photon flux density m^{-2} sec^{-1} at plant height), and a 70% RH. The hydroponic solution was renewed every second day. After 5 days, five uniform seedlings were selected and each was transferred into a single growing unit containing the same nutrient solution with coumarin added at concentrations of 0, 25, 100, and 400 μM for 48 hr. All reagents used were analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO).

Root Morphology. At each coumarin concentration, roots and shoots of each seedling were harvested. Shoots were dried at 70°C for 48 hr for dry weight measurement. The whole root system, as well as single root types (nodal, seminal, radicle), was spread into a plastic transparent tray filled with 3 mm of water so that individual roots and neighbor lateral roots did not overlap and stick. The roots were imaged by scanning (STD 1600, Regent Instruments Inc., Quebec, Canada) and their length measured by WinRhizo Pro system v. 2002a (Instruments Regent Inc., Quebec, Canada). The number of lateral roots on seminal and radicle roots was counted on the scanned images. Branching density was calculated by the number of laterals (n) divided by the length of seminal or radicle roots (cm). In order to analyze swelling of the subapical root zone, the diameter of the first centimeter of root apicals was measured in 1-mm segments. The swelling of each root type and coumarin concentration was calculated by a macro of Sigma Plot v. 7.0 software (SPSS Inc., Evanston, USA) that integrates the area under the plot of the graph using the trapezoidal rule. Finally, single roots were dried at 70°C for 48 hr, and their dry weight was measured.

Nomenclature. According to McCully (1999), the maize root system is based upon a seminal and nodal skeleton upon which the rest of the root system develops through lateral branching of different orders (Figure 1). The seminal skeleton is composed of the radicle, the earliest seed-derived root, and horizontally growing seminal roots. The nodal skeleton emerges from the stem.

Statistical Analysis. The effect of coumarin on total root length was evaluated by analysis of variance with a Tukey test ($P \leq 0.05$). Inhibition of the length of radicle, seminal, and nodal roots by different coumarin concentrations was

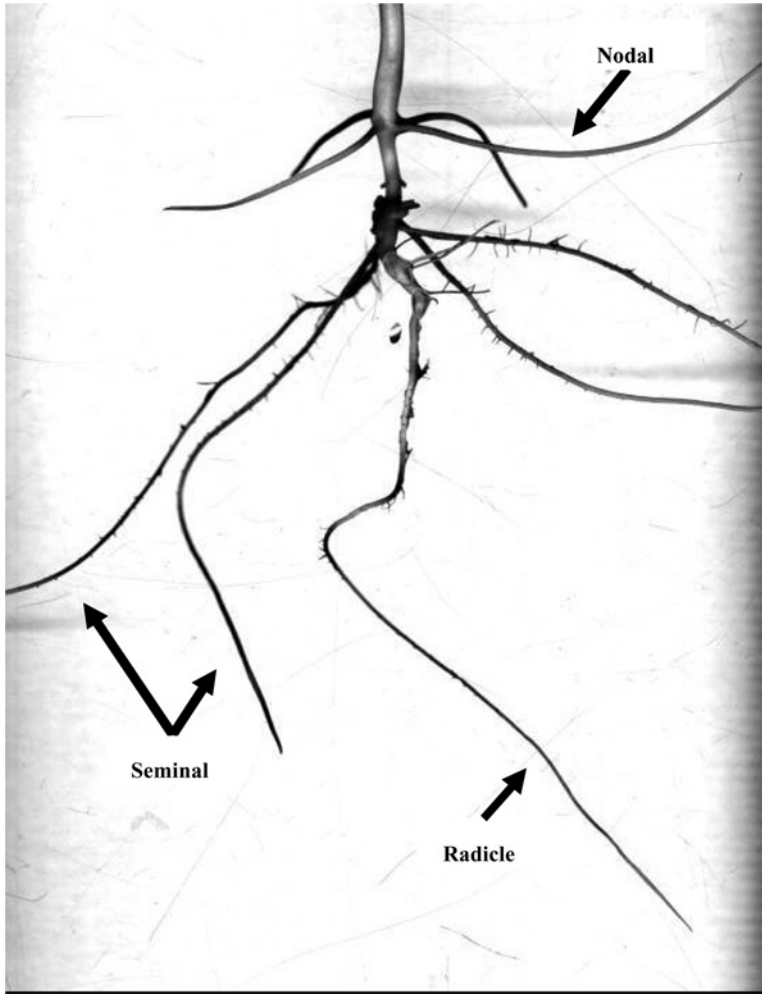

FIG. 1. Structure and nomenclature of the maize root system (after McCully, 1999).

analyzed by non-linear regression using the following equation:

$$y = a \times e^{(-k \times x)}, \tag{1}$$

where y (cm) is the root length, a (cm) is the root length at 0 μM coumarin, k (μM^{-1}) is the rate constant, and x (μM) is the coumarin concentration. The coumarin concentration causing a 50% reduction in root length was calculated as

$$C_{1/2}\ (\mu\text{M}) = \ln(2 \times k^{-1}). \tag{2}$$

Regression parameters (a, k) for each root type were estimated by the method of Pearson VII Limit Minimization (Table Curve 2D v. 4.0 software, Jandel Scientific, Ekrath, Germany) using the Levenberg–Marquardt algorithm, and then compared for the different root types by one-way analysis of variance with a Tukey test ($P < 0.05$). The effects of coumarin on the number of lateral roots, branching density, and dry weight of different root types were subjected to a two-factorial analysis of variance with a Tukey test ($P \leq 0.05$). The effect of coumarin on the diameter of each 1-mm segment of the different root types was evaluated by three-factorial analysis of variance with a Tukey test ($P < 0.05$). All data were evaluated for normality by a Kolmogorov–Smirnov test, and for homoscedasticity by a Levene Median test. Statistical analyses were carried out using Systat v. 8.0 (SPSS Inc., Evanston, USA).

RESULTS AND DISCUSSION

The most frequently reported effects of coumarin on plants are inhibition of root growth and modification of root morphology and histology (Goodwin and Taves, 1950; Svensson, 1971; Aliotta et al., 1993; Kupidlowska et al., 1994; Abenavoli et al., 2001a). Our results showed that coumarin reduced the length

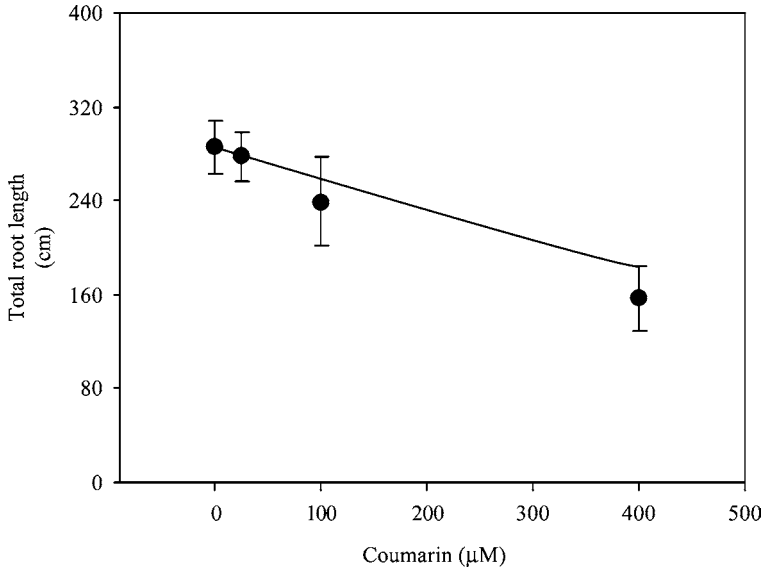

FIG. 2. Length of whole root system of maize seedlings exposed to different coumarin concentrations for 48 hr. Bars represent the standard error ($N = 5$).

of the whole root system of maize seedlings, with a maximum inhibition of 45% of control at the highest concentration tested (400 μM) (Figure 2). This result, obtained on the entire root system, however, does not give information on the specific response of different root types to coumarin. Differences among various root types are more evident under stress (Waisel and Eshel, 2002). For this purpose, we used digital technology, the root image analysis, to measure the morphology of single root types and to understand the specific contribution of root types to the total behavior of the root system. This technology is faster and more accurate than conventional methods. The quantitative evaluation of coumarin on morphologies of individual root components in the maize root system showed varying degrees of sensitivity among nodal, seminal, and radicle roots (Figure 3). The calculated $C_{1/2}$ values revealed that 6 and 1 mM coumarin were necessary to cause a 50% reduction in the length of radicle and seminal root (Table 1), while 0.25 mM coumarin was sufficient to cause the same effect on nodal roots. Based on these results, the following order of sensitivity to coumarin among maize root types was established: nodal > seminal > radicle. An identical order of sensitivity was observed for swelling of the subapical root zone (Table 2, Figure 4).

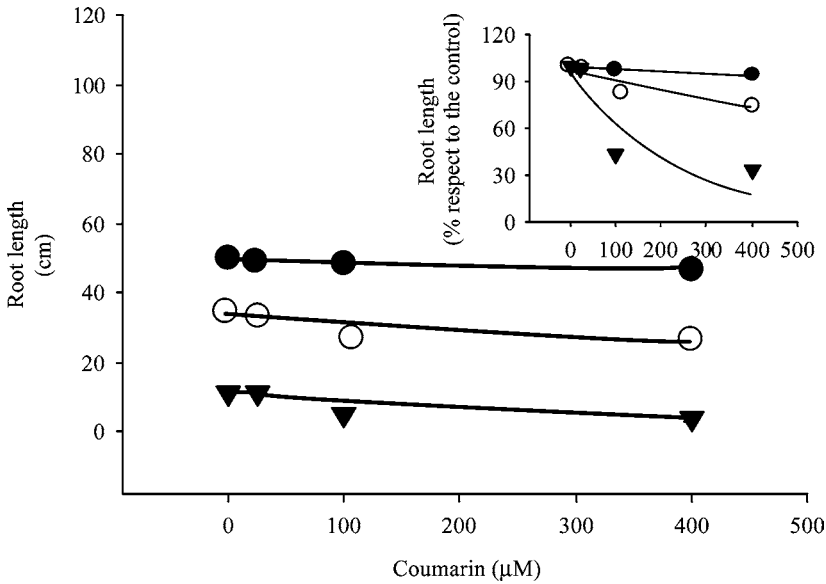

FIG. 3. Length of diverse root types of maize seedlings exposed to different coumarin concentrations for 48 hr. (●) radicle, (○) seminal, and (▼) nodal roots. Bars represent the standard error ($N = 5$). The inset shows the length, in % of untreated control, of diverse root types; symbols as above.

TABLE 1. PARAMETERS RELATIVE TO THE VARIATION OF THE LENGTH OF RADICLE, SEMINAL, AND NODAL ROOTS OF MAIZE SEEDLINGS (7-DAY-OLD) EXPOSED TO COUMARIN FOR 48 HR

Parameters[a]	Radicle	Seminal	Nodal
a (cm)	49.20 a[b] (0.39)[c]	33.80 b (1.89)	11.39 c (2.01)
k (μM^{-1})	0.00010 a (0.00001)	0.00067 b (0.00005)	0.00270 c (0.00020)
$C_{1/2}$ (μM)	6931 a (726)	1034 b (79)	256 c (32)
r^{2d}	0.775	0.632	0.675

[a] Estimated by nonlinear regression according to $y = a \times e^{(-k \times x)}$ whereby a (cm) is the root length at 0 μM coumarin, k (μM^{-1}) is the rate constant, $C_{1/2}$ (μM) represents the coumarin concentration causing a 50% inhibition in root length.
[b] Small letters indicate significant differences (Tukey test, $P < 0.05$).
[c] Values in brackets indicate the standard error ($N = 5$).
[d] r^2 is the coefficient of determination for the nonlinear curve fitting.

Swelling of roots is a typical effect of coumarin and was previously reported by Svensson (1971) and Abenavoli et al. (2001a). This effect was only observed at 400 μM coumarin on all root types of maize seedlings, while negligible effects were observed with other concentrations (Table 2, Figure 4). However, at this concentration, the nodal root axis showed swelling that persisted up to 9 mm from the tip, while it was retained up to 6 mm on the radicle and seminal roots (Figure 4). At 400 μM of coumarin, nodal, seminal, and radicle root swelling was 42, 34, and 31%, respectively, compared with untreated control (Figure 4). Furthermore, the highest coumarin concentration (400 μM) caused transversal cracks in the cortex only in nodal roots, while no cracks were observed in seminal and radicle roots (Figure 5). This suggests the assumption that nodal roots are the most sensitive part of the maize root system to coumarin. Our results reveal for the first time, that

TABLE 2. ANALYSIS OF VARIANCE FOR ROOT DIAMETER ALONG THE ROOT AXES (DISTANCE EFFECT) OF DIFFERENT ROOT TYPES (ROOT TYPE EFFECT) OF MAIZE SEEDLINGS (7-DAY-OLD) EXPOSED TO DIFFERENT COUMARIN CONCENTRATIONS (CONCENTRATION EFFECT)

Effect	df[a]	Mean square	F value[b]
Concentration	3	4.188	200.112***
Root type	2	0.686	32.792***
Distance	9	0.283	13.524***
Concentration × root type	6	0.362	17.274***
Concentration × distance	27	0.209	9.988***

[a] df = degrees of freedom.
[b] Level of significance: *** = significant at $P < 0.001$.

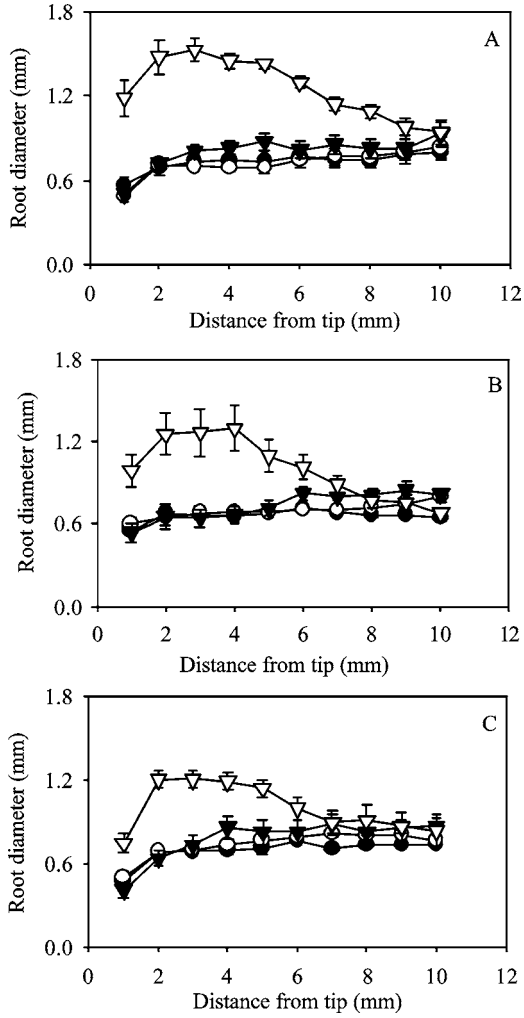

FIG. 4. Diameter of each 1 mm of root segment starting from the tip of different root types (A: radicle; B: seminal; C: nodal) of maize seedlings that were exposed to coumarin for 48 hr. (●) 0 μM, (○) 25 μM, (▼) 100 μM, and (▽) 400 μM. Bars represent the standard error ($N = 5$).

coumarin has a root type specific effect on maize seedlings, whereby nodal roots appear much more responsive than other components in the root system.

Several mechanisms could be responsible for the observed differences in nodal root sensitivity to coumarin. Svensson (1971) and Kupidlowska et al. (1994) reported that coumarin inhibited cell elongation in all cell layers and that

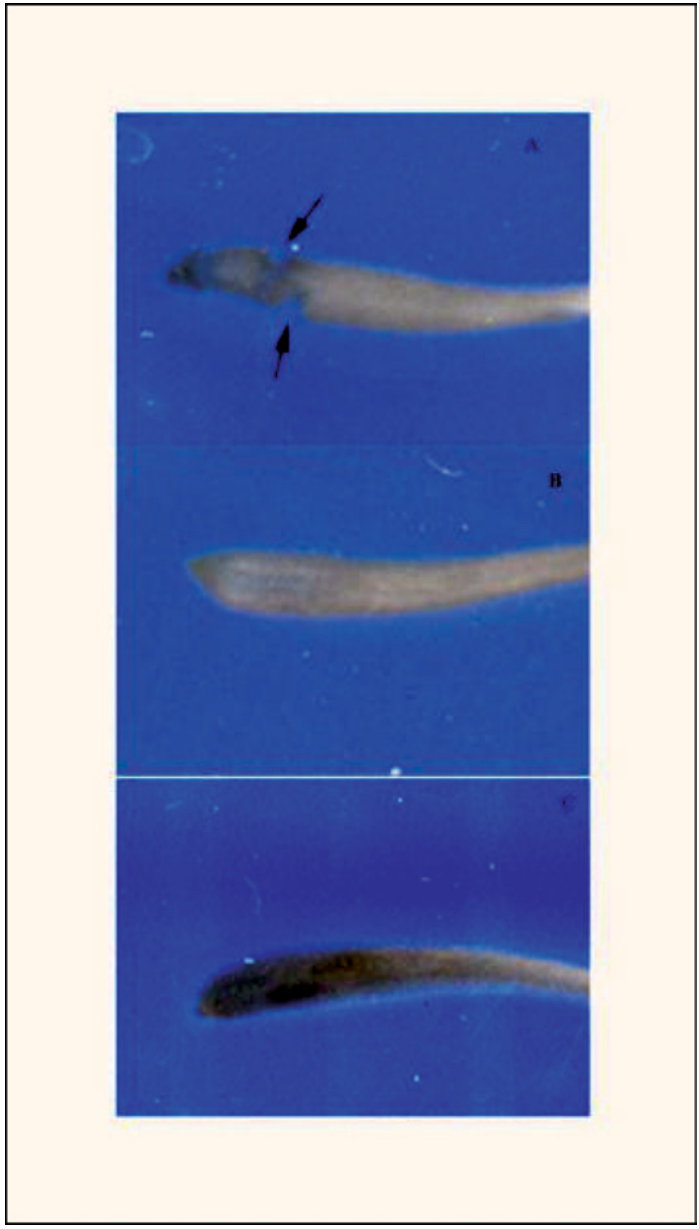

FIG. 5. Apical part of different root types of maize seedlings treated with 400 μM of coumarin for 48 hr. (A) nodal, (B) seminal, and (C) radicle roots. Arrows indicate the transversal crack on nodal root.

TABLE 3. ANALYSIS OF VARIANCE FOR DRY WEIGHT OF NODAL, SEMINAL, AND RADICLE ROOTS, FOR NUMBER OF LATERAL ROOTS, AND BRANCHING DENSITY OF SEMINAL AND RADICLE ROOTS (ROOT TYPE EFFECT) OF MAIZE SEEDLINGS (7-DAY-OLD) EXPOSED TO 0 AND 400 μM COUMARIN (CONCENTRATION EFFECT)

Parameter ($N = 5$)	Concentration	Root type	Concentration × root type
Number of laterals	12.487*[a]	107.885***	1.756[ns]
Branching density	8.378*	23.328***	1.056[ns]
Dry weight	3.802[ns]	53.838***	0.632[ns]

[a] Numbers = F value with level of significance.
* = significant at $P < 0.05$; *** = significant at $P < 0.001$; ns = not significant at $P > 0.05$.

perivascular zones were more sensitive at high concentrations. The sensitivity of maize nodal roots to coumarin exposure could be explained by the narrower perivascular tissue compared with seminal and radicle roots (Luxovà and Kozinka, 1970; McCully, 1999). Forty-eight hours were probably sufficient to inhibit root cell length and cause a crack formation in the nodal root, while the radicle and seminal ones may require longer exposure to the 400 μM coumarin for these effects to manifest.

Nodal roots represent younger root structures than seminal or radicle roots, and could have an increased demand for respiratory energy and ion uptake. Coumarin inhibits the rate of root respiration (Kupidlowska et al., 1994; Abenavoli et al., 2001a), which would preferentially affect the nodal roots with a higher energy demand than either the seminal or radicle roots.

Coumarin decreased the number of lateral roots and the branching density in both seminal and radicle roots compared with the untreated control (Table 3, Figure 6). For seminal and radicle roots, a reduction of 69 and 14% was observed for the number of laterals and 60 and 18% for the branching density, respectively.

Although coumarin modified the growth of different root types in terms of shape, dry weight variation was not observed (Figure 7). In fact, coumarin did not affect the biomass allocation within the maize root system compared to untreated controls [no significant interaction between concentration and root type (Table 3, Figure 7)]. Similar results were obtained for maize seedlings treated with terpenes, another group of secondary metabolites involved in allelopathy (Abrahim et al., 2000). Furthermore, considering that coumarin did not affect shoot dry weight (data not shown), we suspect that time of exposure and/or coumarin concentration had little negative impact on plant metabolism.

Overall, the individual components of the 7-day-old maize root system showed a different sensitivity to coumarin exposure. Shallower, horizontally growing nodal roots were inhibited more by coumarin than deeper growing root types,

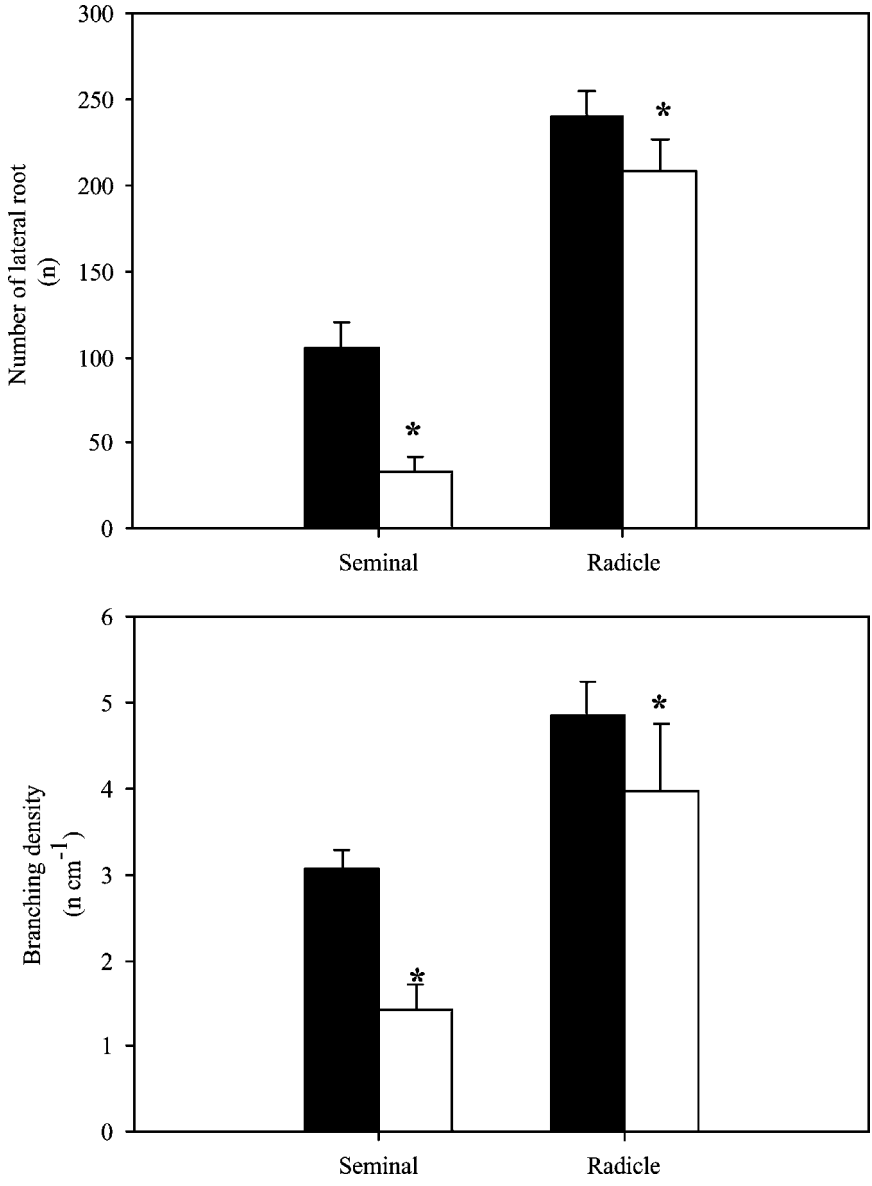

FIG. 6. Number of laterals and branching density of seminal and radicle roots of maize seedlings exposed to coumarin for 48 hr. (■) 0 μM and (□) 400 μM coumarin. Bars represent the standard error ($N = 5$). Asterisks indicate a significant difference from control (Tukey test, $P < 0.05$).

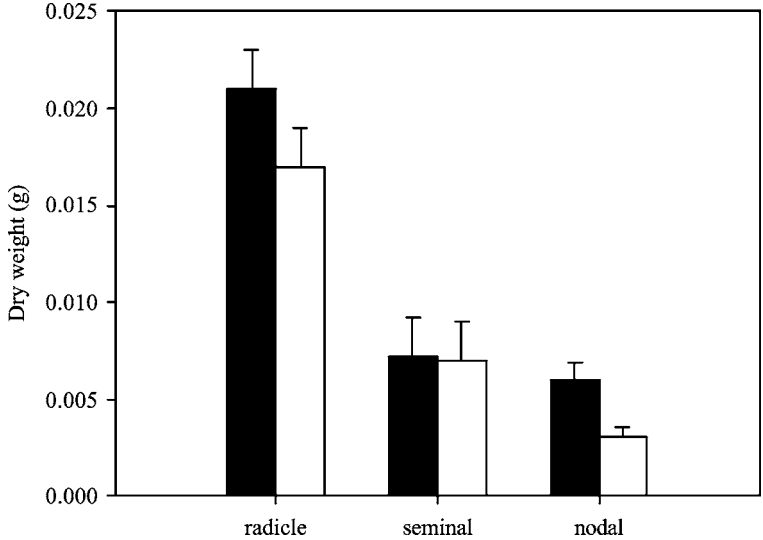

FIG. 7. Dry weight of different root types of maize seedlings exposed to coumarin for 48 hr. (■) 0 μM and (□) 400 μM coumarin. Bars represent the standard error ($N = 5$). Asterisks indicate a significant difference from control (Tukey test, $P < 0.05$).

i.e., radicle and seminals. Considering that allelochemicals are spatially concentrated in top soil layers (Kuiters and Sarink, 1986) where the sensitive nodal roots generally grow, coumarin could have an important ecological impact. The selective effect, along with the spatial soil distribution, could preferentially promote taprooted species over fibrous rooted plants and, consequently, influence the composition of organisms within a plant community.

Acknowledgments—Financial support was provided by the Italian Ministry of Education, University and research PRIN 2002 (project ex-40%).

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PHYTOECDYSTEROIDS: A NOVEL DEFENSE AGAINST PLANT-PARASITIC NEMATODES

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Abstract—The phytoecdysteroid, 20-hydroxyecdysone (20E), is a major molting hormone of invertebrates, possibly including nematodes. As 20E is inducible in spinach, the defensive role against plant-parasitic nematodes was investigated. The effects of direct application on nematodes was assessed by treating cereal cyst nematode, *Heterodera avenae*, juveniles with concentrations of 20E from 8.2×10^{-8} to 5.2×10^{-5} M before applying to *Triticum aestivum* growing in sand. *H. avenae*, *Heterodera schachtii* (sugarbeet cyst nematode), *Meloidogyne javanica* (root-knot nematode), and *Pratylenchus neglectus* (root lesion nematode) were treated with 5.2×10^{-5} 20E and incubated in moist sand. To test the protective effects of 20E in plants, the latter three nematodes were applied to *Spinacia oleracea* in which elevated concentrations of 20E had been induced by methyl jasmonate. Abnormal molting, immobility, reduced invasion, impaired development, and death occurred in nematodes exposed to 20E either directly at concentration above 4.2×10^{-7} M or in plants. Phytoecdysteroid was found to protect spinach from plant-parasitic nematodes and may confer a mechanism for nematode resistance.

Key Words—Phytoecdysteroids, 20-hydroxyecdysone, methyl jasmonate, plant defense, *Spinacia oleracea*, *Heterodera avenae*, *Heterodera schachtii*, *Meloidogyne javanica*, *Pratylenchus neglectus*, nematode.

INTRODUCTION

The impact of plant-parasitic nematodes on global food production is well recognized. Efforts to control this major pest group include selection, breeding, and,

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more recently, molecular engineering for host resistance along with cultural, chemical, and biological methods (Whitehead, 1998). Host resistance has proven to be an economic and environmentally attractive approach applicable to any scale of production from subsistence farming to highly mechanized broad-acre farming. Despite the identification, incorporation, genetic characterization, and application of nematode resistance genes, the biochemical basis of nematode resistance remains largely unknown (Roberts et al., 1998).

Several secondary metabolites are induced in plants following nematode invasion (Zacheo and Bleve-Zacheo, 1995). The responses directly induced include synthesis of phytoalexins, production of hydrolytic enzymes, rapid modification of existing cell wall materials, and systemic responses such as accumulation of proteinase inhibitors (Cramer et al., 1993). However, beside the phytoallexin, glyceollin, a convincing link to mechanisms of nematode resistance remains to be demonstrated for these nematode induced responses in plants. Glucosinolates in *Brassica* provide resistance to plant-parasitic nematodes (Potter et al., 1999). However, these compounds are enzymatically converted to broad-spectrum biocides, not specific to nematodes, and are largely restricted to Brassicaceae.

Ecdysteroids are highly polar steroidal hormones responsible for growth and development in many arthropods. Surveys conducted after their discovery in plants (termed phytoecdysteroid) along with numerous biologically active analogues (Galbraith and Horn, 1966; Nakanishi et al., 1966) indicated that phytoecdysteroids, a diverse group, may be present in a wide range of plants (Dinan and Sehnal, 1995), including many used for food (Imai et al., 1969). Phytoecdysteroids induce abnormal molting in many arthropods with lethal effect (Robbins et al., 1970; Bergamasco and Horn, 1983; Kubo et al., 1983; Lafont et al., 1991; Dinan, 2001). The observation that phytoecdysteroid concentrations increase in response to mechanical damage, insect herbivory, and application of wound hormone, methyl jasmonate, (Schmelz et al., 1999) is consistent with the notion that phytoecdysteroid induction can protect plants from insect attack (Schmelz et al., 2002).

As nematodes are placed in a clade of molting metazoans, the Ecdysozoa, it is likely they all have similar hormonal regulation of ecdysis (Aguinaldo et al., 1997). Since ecdysteroids possess biological activity in free-living and animal nematodes (Rogers, 1973; Dennis, 1976, 1977; Mendis et al., 1983; Nelson and Riddle, 1984; Fleming, 1985; Davies and Fisher, 1994), phytoecdysteroids may also provide an important plant defense against nematodes and, thus, may serve as a basis for the development of resistant cultivars.

We, therefore, examined the effects of 20-hydroxyecdysone (20E), a major (Lafont et al., 1991; Schmelz et al., 1999; Dinan, 2001) and stable (Schmelz et al., 2000) ecdysteroid inducible (Schmelz et al., 1999) in spinach, on migratory (*Pratylenchus*) and sedentary (*Heterodera* and *Meloidogyne*) endoparasitic nematodes in genera that are responsible for most economic damage, by direct application

and through exposure within plants. Spinach was used as a model plant because elevated levels of 20E can be induced (Schmelz et al., 1999) and it is parasitized by species of the important phytophagous nematode genera. Moreover, wound-induced root 20E accumulations were found to be the result of increased *de novo* 20E synthesis in spinach roots (Schmelz et al., 1999), that may confer enhanced resistance to nematodes. Since spinach also contains a minor phytoecdysteroid, polypodine B, the effect of this compound applied exogenously on *Heterodera avenae* was determined. The ability of nematodes to induce 20E in plants was also assessed.

METHODS AND MATERIALS

Exogenous Ecdysteroid. Second stage juveniles (J2) of *Heterodera avenae* (cereal cyst nematode), hatched from eggs in soil organic matter containing cysts incubated in Whitehead trays (Whitehead and Hemming, 1965) at 10°C, were treated with different concentrations of 20E and polypodine B to examine the effect on invasion and development in wheat (*Triticum aestivum*). *H. avenae* was used because a uniform invasive stage can be easily obtained and only one life cycle is completed in wheat. The nematodes were incubated for 24 hr at 15°C in 2-ml of the following concentrations of 20E (Sigma, 98.8% purity) in 10% methanol: 8.2×10^{-8}, 4.2×10^{-7}, 2.1×10^{-6}, 1.0×10^{-5}, and 5.2×10^{-5} M. Some nematodes were also treated with 2-ml 5.2×10^{-5} M polypodine B (Sigma, 95% purity) and incubated under the same conditions. Both water and 10% methanol controls were included. After treatment, nematodes were rinsed with three changes of water. The 20E treated and controls were visually examined for vitality before 600 J2/plant were applied to 10-day old wheat cv. Egret seedlings grown hydroponically in 250-ml pots containing pasteurized sand. Wheat was used because it did not contain any inducible methanol-extractable or ecdysteroid-like compounds. Plants were maintained by adding 50 ml/plant full strength Hoagland solution (Hoagland and Arnon, 1938) every other day in a growth room (15°C; 85% RH; 12 hr L:D) (Mor et al., 1992). Nematodes were extracted from the sand by flotation and a sieving method (Byrd et al., 1966), stained in roots, and were counted 30 days after inoculation. Molting nematodes were also recorded and tested for vitality (Shepherd, 1962) by staining with 0.01% new blue R (GT Gurr, Ltd.).

Mixed stages of *Pratylenchus neglectus* (root lesion nematode) and J2 of three sedentary endoparasitic nematodes, *Meloidogyne javanica* (root-knot nematode), *H. avenae*, and *Heterodera schachtii* (beet cyst nematode) were treated with 2-ml 5.2×10^{-5} M 20E. Nematodes were incubated for 24 hr at 15°C, except for *M. javanica,* which was kept at 25°C. *P. neglectus*, a migratory endoparasitic nematode, was cultured axenically in carrot callus using a modified protocol of Moody et al. (1973). *M. javanica* and *H. schachtii* J2 were hatched at 25°C from eggs

from greenhouse cultures in tomato and silverbeet, respectively. Known numbers of the treated nematodes were dispensed into 100-g sand in 70-ml polystyrene screw cap containers after rinsing them as described above. Each container was watered to and maintained at field capacity after adding 300 nematodes/container. Nematodes from each container were extracted from the sand by flotation and sieving (Byrd et al., 1966) and evaluated for vitality and abnormal molting after incubation for 2 wk.

Endogenous Phytoecdysteroid. The effect of endogenous 20E in spinach (*Spinacia oleracea* cv. Avon) on the three species of nematodes was assessed. Spinach was germinated and grown in seed trays for 11 days before being transplanted into 300-ml pots to grow hydroponically in pasteurized sand. Hoagland solution (full strength) was applied every other day at the rate of 50 ml/plant. To induce 20E production, plants were drenched with 10-ml 10^{-4} M methyl jasmonate 15 days after transplanting (referred to as "treated plants") because jasmonates induce *de novo* ecdysteroid synthesis in the roots (Schmelz et al., 1999). Three days following induction, treated and untreated plants were inoculated with *H. schachtii*, *P. neglectus*, and *M. javanica* (600 nematodes/plant). Uninoculated controls of both induced and untreated plants were included. Plants from each treatment were sampled, and roots were washed thoroughly 5 and 10 days after inoculation. Nematodes were extracted and counted from both the sand and the roots as described above. Molting nematodes were recorded and tested for vitality. Additional plants were harvested 30 days after inoculation to determine fresh and dry weights of shoots and roots.

Phytoecdysteroid Quantification. Quantification of 20E in shoot and roots, as described in Schmelz et al. (1999), used reverse-phase high performance liquid chromatography (RP-HPLC) with a C-18 column (Waters Sperisorb ODS-2, 4.6 mm $\times$ 150 mm, 5-μm particle column). Isocratic elution was performed with water-methanol (55:45 v/v) as mobile phase at 1 ml/min at room temperature with detection at UV 254 nm, 0.1 AUFS.

Experimental Design and Statistical Analysis. Plants of uniform height and vigor were arranged in a randomized complete block design with 10 replicates before treatments in both experiments were applied. Analysis of variance was used to analyze the data and means were compared by using Tukey's test.

RESULTS

Effect of Exogenous Ecdysteroid. Immediately following nematode treatment and just before inoculation, no effect of 20E on the condition of *H. avenae* J2 was evident. Also, water and 10% methanol controls did not differ significantly in any parameter measured, so only 10% methanol controls are presented. After 30 days, the total number of treated nematodes that failed to invade the plants increased

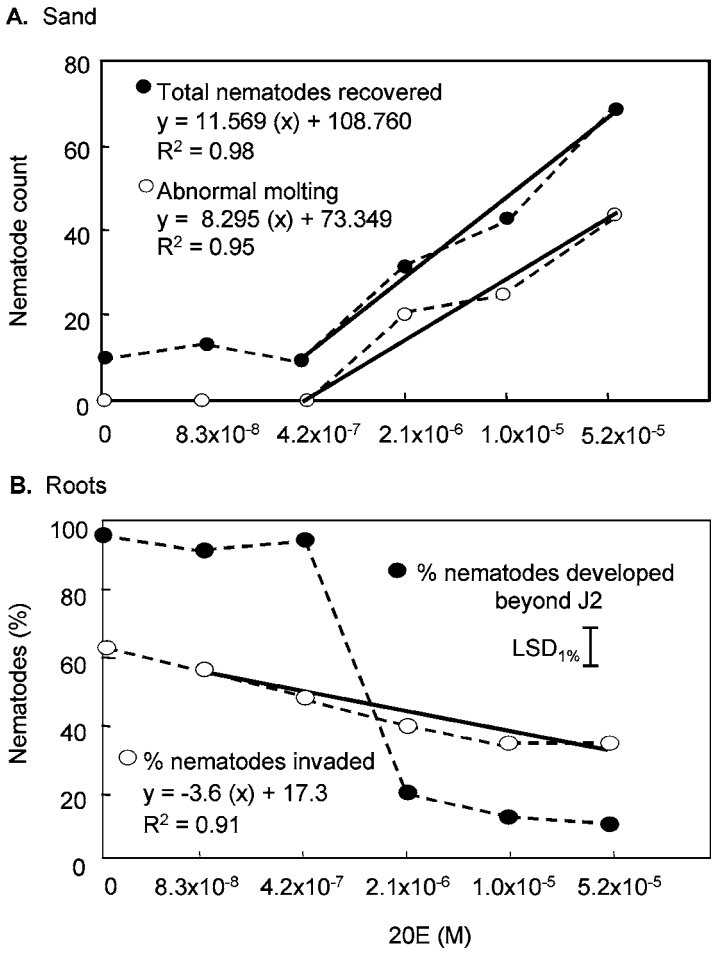

FIG. 1. Effect of exogenous 20-hydroxyecdysone (20E) on invasion by and development of *Heterodera avenae* in wheat cv. Egret 30 days after inoculation following treatment of the nematodes with varying concentrations of 20E in 10% methanol. A. Invasive stage (J2) of *H. avenae* recovered from sand and the number exhibiting abnormal molting. B. Percentage of inoculated nematodes that invaded roots and percentage of invading nematodes that developed beyond the invasive stage.

linearly ($r^2 = 0.98$) with 20E concentration above 4.2×10^{-7}M (Figure 1). The number of *H. avenae* with adverse morphological defects in the sand increased linearly ($r^2 = 0.95$) with 20E concentration above 4.2×10^{-7}M (Figure 1). Exposure to 20E caused incomplete apolysis, shortening of juveniles, and loosening of

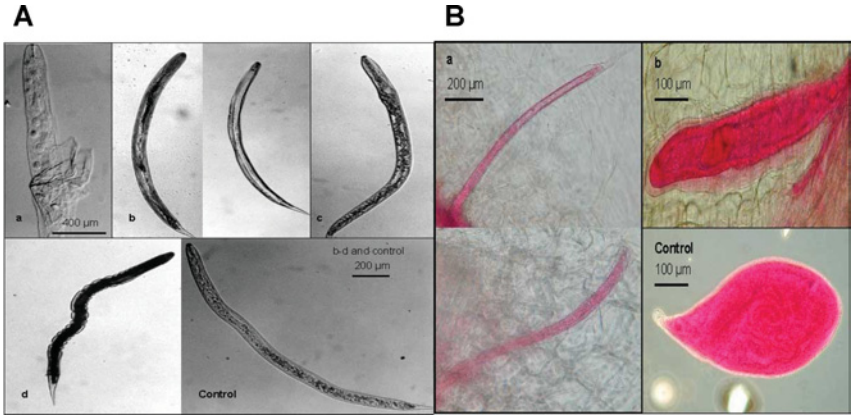

FIG. 2. Detrimental effects of exogenous 20E on *Heterodera avenae* including A. incomplete apolysis (a), reduction in size (b), partial bloating (c), and random detachment of cuticle (d) of invasive stage from the sand, and B. abnormal molting of invasive stage (a) and third stage juveniles (b) in wheat cv. Egret roots 30 days after inoculation.

their cuticle sections in those recovered from sand, as well as in those that invaded roots (Figure 2). The number of nematodes in the roots decreased ($r^2 = 0.91$) with increasing concentration of 20E, and the proportion that developed beyond the invasive stage was reduced to less than 20% when treated with 2.1×10^{-6} M 20E or more ($P < 0.01$, Figure 1). In contrast, equivalent numbers of polypodine B treated (64%) and untreated (62%) nematodes invaded the wheat roots and development to adults was largely unimpaired.

In the subsequent experiment in which four nematode species were treated with 5.2×10^{-5}M 20E, abnormal molting and mortality was observed in *H. avenae*, *H. schachtii*, and *P. neglectus* (Figure 3). In contrast, for *M. javanica*, only increased mortality was observed with most juveniles being immobilized by the treatment even before inoculation. Since the effects of water and 10% methanol controls did not differ significantly, only the 10% methanol control was used for comparison.

In the case of the two *Heterodera* species (Figure 3), about 26% of the treated nematodes molted abnormally, and 80% or more were dead (as shown by vitality staining). In the control, no abnormal molting was observed, and only 5% of the nematodes were dead. Treatment of *P. neglectus* resulted in 58% abnormal molting compared to 12% in the control (Figure 3). Again, about 80% of treated nematodes died compared to 15% in the control. Likewise, 80% of the treated *M. javanica* recovered were dead compared to only 15% in the control. In all cases, the treatment effects differed from the respective controls by probabilities less than 0.01.

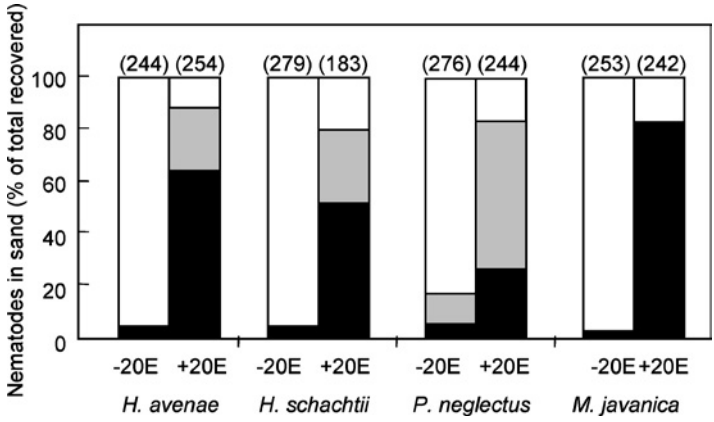

FIG. 3. Proportion (%) of active (white; LSD 1% = 48.1), abnormally molting (shaded; LSD 1% = 31.5), and immobile/dead (black; LSD 1% = 52.9) *H. avenae, H. schachtii*, *P. neglectus*, and *M. javanica* after 2 wk of incubation in moist sand following treatment with (+20E) and without (−20E) 20E; total number of nematodes recovered shown in brackets. LSD = least significant difference

Effect of Endogenous Ecdysteroid. Compared to the control or uninduced/uninoculated plants, treatment of spinach with MJ resulted in increased concentrations of 20E in the roots (78%, $P < 0.05$) and shoots (61%, $P < 0.01$), and affected the invasion and development of the nematodes applied. No other methanol extractable compound was induced by detection at 254 nm. MJ treatment did not affect plant growth.

In the case of *H. schachtii*, induction with MJ had no effect on the number of nematodes found in the sand or the roots (Figure 4). However, 5 days after inoculation, 54% of the nematodes that invaded roots of treated plants were molting prematurely, but none were in untreated plants (Figure 4). Ten days after inoculation, normal molting was detected in untreated plants, but 32% more *H. schachtii* were molting in the treated plants (Figure 4).

Five days after inoculation of untreated plants, *H. schachtii* increased 20E concentration in the shoots and roots by 250% compared to the untreated/uninoculated control (Figure 5A). This concentration was equivalent to that induced by MJ treatment. Induction by combination of MJ treatment and inoculation, however, did not increase 20E concentration over that of inoculation alone.

Elevating 20E before inoculation protected spinach from nematode damage, resulting in growth that was equivalent to uninoculated controls. Inoculation of untreated plants reduced dry root and shoot mass by 53% and 56% (Figure 5B), respectively, compared to untreated/uninoculated and treated/inoculated spinach.

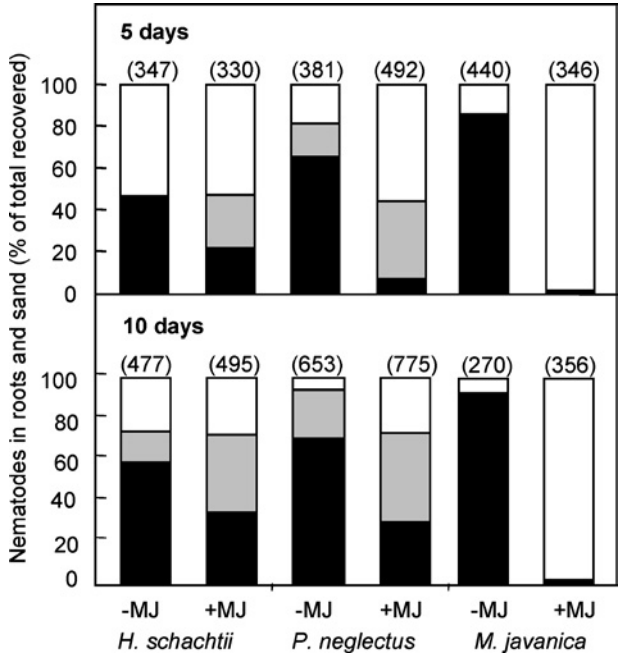

FIG. 4. Effect of induction of 20E in spinach cv. Avon by methyl jasmonate (MJ) on the proportion (%) of total nematodes recovered from the sand (white; −MJ: LSD = 16.18, 5 days; 11.77, 10 days; +MJ: LSD = 18.13, 5 days; 23.59, 10 days) and roots (normal, black; LSD = 29.92, 5 days; 32.95, 10 days; molting, shaded; LSD = 15.61, 5 days; 27.91, 10 days) 5 and 10 days after inoculation of *H. schachtii*, *P. neglectus*, and *M. javanica*, in treated (+MJ) and untreated (−MJ) spinach; numbers in brackets are total number of nematodes recovered.

In the case of *P. neglectus*, greater numbers of nematodes were recovered from the sand of treated plants (37% and 28% of inoculum applied 5 and 10 days after inoculation, respectively, $P < 0.01$, Figure 4), but there was no effect on the number found in the roots (Figure 4). However, the proportion of molting nematodes had increased ($P < 0.01$) in treated plants (Figure 4). Only normal molting was found among nematodes in untreated plants. By contrast, in treated plants, a large proportion of nematodes of all stages molted abnormally.

Five days after inoculation of untreated plants, *P. neglectus* had induced a 250% and 500% increase in 20E in shoots and roots, respectively, compared to the untreated/uninoculated control (Figure 5A). The induction of 20E by *P. neglectus* inoculation was equivalent to that induced by MJ treatment, but again combined treatment and inoculation did not increase 20E concentration over that of inoculation alone.

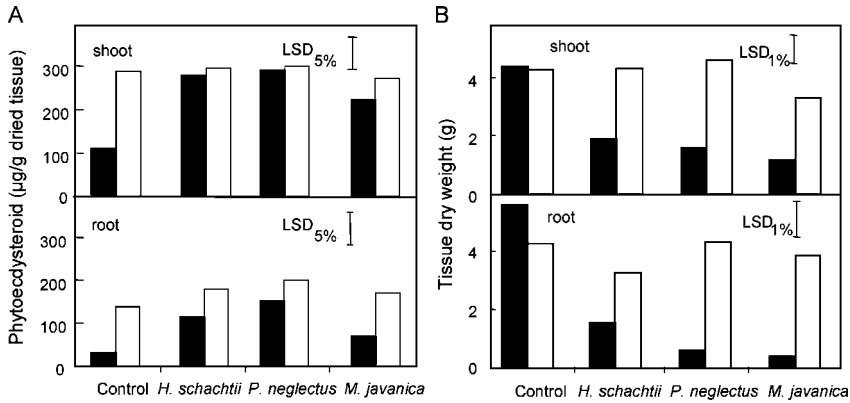

FIG. 5. A. Concentration of 20E in shoots and roots of methyl jasmonate-induced (white) and untreated (black) spinach 10 days after inoculation of *Meloidogyne javanica*, *Pratylenchus neglectus*, and *Heterodera schachtii*. B. Shoot and root dry mass of methyl jasmonate-induced (white) and untreated (black) spinach 10 days after inoculation of *Meloidogyne javanica*, *Pratylenchus neglectus*, and *Heterodera schachtii*.

Inoculation of untreated spinach with *P. neglectus* reduced root and shoot dry weight 86% and 65%, respectively ($P < 0.01$), compared to untreated and uninoculated controls (Figure 5B). In contrast, preinoculation induction of 20E protected the plant from damage.

In the final case, greater numbers of *M. javanica* applied were also recovered from the sand of induced plants (47% and 55%, 5 and 10 days after inoculation, respectively, $P < 0.01$, Figure 4), but there was no effect on the number found in roots (Figure 4). However, only 1% and 2% of the *M. javanica* inoculated were found in the treated spinach roots, compared to 63% and 42% in untreated plants 5 and 10 days after inoculation, respectively (Figure 4). No morphological abnormalities were observed in nematodes from the sand or roots. Molting nematodes were only observed in untreated plants and they appeared normal. Molting was not observed in nematodes from the sand.

Five days after inoculation of untreated plants, *M. javanica* had induced a 58% and 50% increase in 20E in roots and shoots, respectively, compared to the untreated/uninoculated control (Figure 5A). The concentration of 20E induced by *M. javanica* inoculation alone was less than that induced by MJ, but still greater than the untreated controls ($P < 0.05$).

Inoculation of untreated spinach with *M. javanica* damaged plants, reducing root and shoot dry weight by 90% and 65%, respectively ($P < 0.01$) compared to untreated/uninoculated controls (Figure 5B). Damage was sufficient to cause death of some plants. *M. javanica* inoculation did not cause damage to MJ treated plants.

DISCUSSION

The first part of this study demonstrated biological activity of exogenous 20E in plant-parasitic nematodes. The most evident physiological response was abnormal molting resulting in immobility and death or in the case of *M. javanica*, just immobility and death. In *H. avenae*, the consequence was impaired root invasion and development. *Heterodera* second stage juveniles molt only after invasion of the roots (Wyss and Zunke, 1986), so premature molting of the invasive stage is clearly disruptive. All stages of *Pratylenchus* survive in the sand and, apart from eggs, can invade roots. However, increased and abnormal molting in 20E-treated *P. neglectus* would have likewise affected host invasion.

This is the first evidence of an adverse effect of exogenous 20E on plant-parasitic nematodes, which adds to the few known reports of activity in animal parasites, *viz. Ascaris suum* (Fleming, 1985) at 1×10^{-8} M and *Trichinella spiralis* (Rogers, 1973) at 3.12×10^{-5} M, and the fungal-feeding nematode, *Aphelenchus avenae* (Davies and Fisher, 1994) at 2×10^{-3} M.

The ecdysteroids, recognized as natural hormones regulating molting and metamorphosis in insects (Butenandt and Karlson, 1954), are, thus, biologically active in similar ways in nematodes. Whether the ecdysteroids of insects are the same or similar to the molting hormones regulating similar physiological processes in nematodes is unknown. However, the close parallel of their physiological action indicates that they might share similar chemistry. The externally applied ecdysteroid concentration required to induce molting and disrupt development in *H. avenae* exceeded expected physiological concentrations. The high polarity of ecdysteroids, however, may severely limit their absorption through the nematode cuticle, and only a minimal increase in internal 20E concentrations might happen. In adult *Dirofilaria immitis*, for instance, ecdysone was sparingly absorbed through the cuticle when the nematode was incubated with the hormone, but once absorbed, it was metabolized readily to several less polar products (Mercer et al., 1989). Nonetheless, molting of the third-stage larvae of *D. immitis* is stimulated by 20E, at both 1×10^{-5} and 1×10^{-7} M (Barker et al., 1990).

The adverse responses to application of ecdysteroid are of greater significance than hormonal function in nematodes. The effects of exogenous 20E indicate a plant defense function, given that phytoecdysteroids occur widely and the biologically active concentrations we found were well within the range occurring in plants (Dinan, 2001).

The effects of endogenous 20E in spinach on the three species of phytophagous nematodes further indicate a plant defense role. Given the similarities with the effects of exogenous application of 20E on the nematodes tested, i.e., abnormal molting of *H. schachtii* and *P. neglectus* and immobilization of *M. javanica* leading to impaired invasion, the ecdysteroid, 20E, is implicated as the principal compound induced by MJ that affects nematodes. Polypodine B, which

is also present in spinach and coeluted with 20E did not affect *H. avenae* when applied as exogenous treatment.

Jasmonic acid induces genes involved in the biosynthesis of compounds that are involved in plant defense (Creelman and Mullet, 1995, 1997; Baldwin, 1998). We are, therefore, not excluding the possibility of other methyl jasmonate inducible compounds in spinach that were not detected by our system. Although methyl jasmonate may have other unmeasured effects, its induction of 20E in high concentrations and the physiological and direct inhibitory effect of 20E on the nematodes are sufficient to account for the protection observed.

The most abundant phytoecdysteroid in plants is 20E, although it may be accompanied by one or two other related major phytoecdysteroids (Lafont et al., 1991; Dinan, 2001). Thus, its occurrence as the predominant phytoecdysteroid in plants as well as in insects, and its induction by both insects and nematodes, supports its defensive role. Schmelz et al. (2000) demonstrated through radioactive labeling of 20E that ecdysteroids are metabolically stable in spinach, which further suggests a role in plant defense.

The results of the study support the findings of Schmelz et al. (1998) that induced accumulations of 20E are caused by active root processes in spinach. The root synthesis of 20E has also been established in other systems (Grebenok and Adler, 1993; Tomas et al., 1993). However, the mechanism responsible for generating induced accumulations of root phytoecdysteroids is unknown, although the modulation of gene expression and *de novo* synthesis has been predicted (Creelman et al., 1992; Gundlach et al., 1992). The exact location of 20E in roots has not been established, but since abnormal molting of *P. neglectus* and *H. schachtii* occurred in the cortical cells, 20E must be present in this tissue. The preinvasion effect of *M. javanica* indicates also that 20E diffuses into the rhizosphere.

In summary, the effects of endogenous 20E, preinoculation induction of 20E in spinach partially inhibited invasion of *P. neglectus* and markedly that of *M. javanica*. Abnormal molting occurred in *H. schachtii* and *P. neglectus*, and development of the two sedentary species (*H. schachtii* and *M. javanica*) was halted. Development of the migratory *P. neglectus* applied as mixed stages, was not directly assessed, but the abnormal molting in this species indicates that impaired development was likely. In all cases, plants were protected from nematode damage. The ability of each of the three nematode species to induce 20E was demonstrated. However, nematode-induced 20E appeared not to have been sufficiently rapid to prevent damage at the inoculation rate used or to affect the nematodes within the period assessed.

The effects of endogenous 20E in spinach on *P. neglectus* and *H. schachtii* paralleled the effects of exogenous 20E. Both nematodes failed to develop in spinach roots because of the onset of physiological abnormalities (abnormal molting) after invasion. The preinoculation induction of 20E did not reduce the invasion

by these nematodes. The intracellular movement of *P. neglectus* (Zunke, 1990) and *H. schachtii* (Wyss and Zunke, 1986) within the root cortex may have exposed them to high 20E concentration in this tissue. *P. neglectus*, an endoparasitic nematode, invades roots randomly at any point and migrates intracellularly (Zunke, 1990). *H. schachtii* also migrates intracellularly, but moves directly toward the differentiating vascular tissue at the root tips where it establishes a permanent feeding site (Wyss and Zunke, 1986). In contrast to *M. javanica*, a sedentary endoparasitic nematode that migrates intercellularly through the cortex causing limited damage (Wyss, et al., 1992), no abnormal molting of the few that invaded the roots was observed. Rather, the invasion of spinach by *M. javanica* was impaired by the induction of 20E, which indicates sensitivity to extracellular 20E and the movement of the ecdysteroid into the rhizosphere. An increased concentration of 20E in the roots appears to protect the plant from *M. javanica*, providing preinvasion resistance, which is uncommon; the majority of recorded resistance occurs post invasion (Kaplan and Keen, 1980; Trudgill, 1991; Niebel et al., 1993; Sijmons, 1993). Immobilization of *M. javanica* preinvasion is consistent with the effect of exogenous 20E on this nematode. It appears that *M. javanica* is more sensitive to 20E than *H. schachtii* and *P. neglectus*, as these are only affected post invasion and develop morphological changes (abnormal molting) before they die.

The uniqueness of the observed defense provided by 20E against plant-parasitic nematodes is that it protects against nematode species with differing parasitic behavior and operates both pre- and postinvasion. Known nematode resistance in crop plants is stage and species specific (Kaplan and Keen, 1980; Trudgill, 1991; Roberts et al., 1998; Opperman, 2001). The 20E defense in spinach imparts tolerance to the three nematodes tested by reducing their damaging impact on yield, and resistance by reducing the nematode capacity to reproduce.

Previous studies have indicated an allelochemical role for phytoecdysteroids as hormonal disruptors and toxins for insects and some other invertebrates (Robbins et al., 1970; Kubo et al., 1983; Arnault and Sláma, 1986; Camps and Coll, 1993; Savolainen et al., 1995; Blackford and Dinan, 1997; Mondy et al., 1997). This study may be the first to implicate phytoecdysteroids as a resistance mechanism against plant-parasitic nematodes, and, thus, adds to the few known induced plant defenses against them (Kaplan and Keen, 1980; Huang and Barker, 1991; Oka et al., 2000; Kempster et al., 2001).

Deliberate induction or breeding for constitutive levels of phytoecdysteroid in economically important plants may be useful for nematode population management.

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A RESISTANT PREDATOR AND ITS TOXIC PREY: PERSISTENCE OF NEWT TOXIN LEADS TO POISONOUS (NOT VENOMOUS) SNAKES

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Abstract—The Common Garter Snake (*Thamnophis sirtalis*) preys upon the Rough-skinned Newt (*Taricha granulosa*), which contains the neurotoxin tetrodotoxin (TTX) in the skin. TTX is toxic, large quantities are present in a newt, and highly resistant snakes have the ability to ingest multiple newts; subsequently snakes harbor significant amounts of active toxin in their own tissues after consuming a newt. Snakes harbor TTX in the liver for 1 mo or more after consuming just one newt, and at least 7 wk after consuming a diet of newts. Three weeks after eating one newt, snakes contained an average of 42 μg of TTX in the liver. This amount could severely incapacitate or kill avian predators, and mammalian predators may be negatively affected as well.

Key Words—*Taricha granulosa*, *Thamnophis sirtalis*, toxicity, resistance, chemical defense, tetrodotoxin, aposematism, coevolution.

INTRODUCTION

Amphibians are characterized by epidermal granular glands that secrete substances with primarily defensive functions (Duellman and Trueb, 1986), some of which are lethal to predators. Yet many snakes have evolved resistance to toxins that kill other predators, such as *Heterodon* and *Xenodon* that eat toads of the genus *Bufo*

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(see Smith and White, 1955), *Liophis* that eat harlequin frogs (*Atelopus sp.*), and poison dart frogs *(Dendrobates* and *Phyllobates*; Myers et al., 1978), and Central American colubrids (*Thamnophis fulvus, Coniophanes fissidens, Pliocercus elapoides*, and *Rhadinaea* spp.) that eat the plethodontid salamander *Bolitoglossa rostrata* (see Brodie and Ducey, 1991). Similarly, the Common Garter Snake (*Thamnophis sirtalis*) preys upon the Rough-skinned Newt (*Taricha granulosa*).

The Rough-skinned Newt contains the powerful neurotoxin tetrodotoxin (TTX) in the skin (Mosher et al., 1964; Hanifin et al., 1999), which blocks voltage-gated sodium channels in nerve and muscle tissue, thereby inhibiting action potentials (Narahasi et al., 1967). TTX is extremely potent; the oral LD_{50} for a mouse is 334 μg/kg (Mosher et al., 1964; Kawasaki et al., 1973). In other words, only 6–7 μg of orally administered TTX will kill 50% of 20 g mice. *T. granulosa* are variable in toxicity throughout their geographic range and can vary greatly within a population as well (Hanifin et al., 1999, 2002). However, in one toxic population (Soap Creek in the Willamette Valley, Benton Co., Oregon), adult newts contain from 0.6 to 13.0 mg of TTX in their skin (Hanifin et al., 2004), an amount of toxin far in excess of that necessary to kill almost any potential predator of *T. granulosa* (Brodie, 1968).

Nonetheless, *T. sirtalis* consume *T. granulosa* on a regular basis (e.g., Brodie, 1968). Resistance to the toxin occurs in certain populations of *T. sirtalis* that prey upon the newt and have apparently coevolved with them (e.g., Brodie and Brodie, 1990, 1999a,b; Geffeney et al., 2002). The outcomes of coevolutionary interactions may vary over the geographic range in species interactions because of gene flow or differences in local selection (Thompson, 2000). Although some locations exhibit newts with little or no toxicity and correspondingly low to nonresistant snakes, other populations of snakes and newts have been caught in an escalation of reciprocal selection on toxicity and resistance that has led to extremely toxic newts and correspondingly highly resistant snakes—hotspots of snake–newt coevolution (Brodie et al., 2002). Benton Co., in the Willamette Valley, Oregon, is one such locality. A captive snake from the Benton Co., Oregon, population consumed eight adult newts in a 2-wk period (Brodie, 1968). Thus, an individual garter snake may ingest phenomenal amounts of toxin and survive.

Because of the extreme toxicity of TTX, the large quantities present in a newt, and the ability of highly resistant snakes to ingest multiple newts, the possibility arises that snakes may harbor significant amounts of active toxin in their own tissues after consuming a newt. Dendrobatid and mantelline frogs acquire toxicity from dietary precursors (Daly et al., 1997; Saporito et al., 2003). A genus of Natricine snake (*Rhabdophis*, closely related to *Thamnophis*) encompasses several species that appear to sequester toad (*Bufo*) toxins, which are then secreted from nuchal glands in the neck region (Akizawa et al., 1985). The snakes exhibit unique antipredator behavior that exposes predators to the glandular secretion (Mori et al., 1996).

Passive or active containment, sequestration, or failure to flush TTX from the system could generate elevated concentrations of the toxin in the tissues of *T. sirtalis*. The possibility arises that snakes may exploit the toxic newts and receive protection, *via* the chemical defense of TTX, from their own predators. Here, we investigate the location and quantities of TTX accumulation in Common Garter Snakes (*T. sirtalis*) after ingestion of Rough-skinned Newts (*T. granulosa*), factors that influence the accumulation of TTX in the snakes, and possible consequences of that accumulation for snake predators.

METHODS AND MATERIALS

Common Garter Snakes (*T. sirtalis*) were fed Rough-skinned Newts (*T. granulosa*), and the fate of the toxin within the snakes was determined. These animals were collected from Benton Co., Oregon, where newts are highly toxic and snakes comparably resistant (Brodie et al., 2002). Snakes were housed in 38-l aquaria, given access to a thermal gradient with an ambient temperature of 25°C, and exposed to a 12:12 L/D cycle. Previous work showed that the majority of toxin exposure to snakes consuming amphibians occurs in the upper gastrointestinal (GI) track (Brodie and Tumbarello, 1978; Williams et al., 2002), and that exposure time (defined as the length of time a newt occupied the upper GI track) influenced the intoxication of a snake (Williams et al., 2003). Thus, snakes were randomly offered live newts for feeding trials.

Feeding Trials and Tissue Collection. Newt snout–vent length (SVL), total length (TL), and mass were recorded immediately before snake-feeding; snake SVL, TL, and mass were taken after the feeding trial. For those snakes that consumed newts, mass was estimated by subtracting the mass of the newt. Snakes were euthanized with 0.5–1.5 ml of 10% chlorotone, or 0.05–0.25 ml Beauthanasia-D, depending on snake mass. In concordance with tissues examined in rats and mice after administration of TTX (Ogura, 1958; Kao, 1966), tissue samples of liver, kidney, heart, skeletal muscle, blood, and musk were collected from each snake. Tissues were immediately frozen at −80°C after collection.

Snakes were assigned to four groups. Group A ($N = 9$) from Benton, Co., OR and Clatsop Co., OR was housed in the lab as above and fed only fish weekly for over 1 year. Snakes in Clatsop Co., OR also exhibit high resistance to TTX, though less so on average than those from Benton Co., OR (Brodie et al., 2002). Group A was offered a diet of newts (two newts/wk for 5 wk). After 5 wk, snakes were returned to a diet of fish. Snakes were sacrificed weekly, and tissues were collected to examine TTX concentrations. Sampling in Group A was expanded to six snakes during the fourth week after the last newt was consumed in order to evaluate variation in toxicity. Group A was sampled before a nonlethal assay for newt toxicity was developed, thus, the quantity of toxin ingested by these snakes is unknown. By

monitoring the decay of TTX in Group A, we were able to evaluate the assumption that 9 mo was enough time for TTX to be purged from wild-caught treatment snakes (Group D below). *T. sirtalis* from Bear Lake Co., Idaho were allopatric with newts, and served as a negative control (Group B; $N = 2$). Snakes in Group C ($N = 6$) from Benton Co., OR had been kept in the lab for 12 mo and fed only fish. They were screened for signs of the toxin in their liver as an additional negative control.

Treatment snakes (Group D; $N = 15$) were fed a diet of fish weekly for at least 9 mo, enabling us to assume that they were TTX-free before treatment. Group D was fed one newt each then returned to a diet of fish. Tissues were collected on a weekly basis by sacrificing snakes; tissues were collected from five snakes 1 wk after newt consumption, four snakes after 2 wk, four snakes after 3 wk, and four snakes after 4 wk. For snakes in Group D only, the mass of the liver, two kidneys combined, and heart were recorded during tissue collection. A skin sample was collected from each newt given to treatment snakes (Group D) in order to later determine newt toxicity.

Before snake-feeding trials, each newt was anesthetized with 3% Tricaine, and a skin-biopsy punch (Acu-Punch™, Acuderm Inc.) was used to extricate a 5-mm diam circle of mid-dorsal skin as per Hanifin et al. (2002). After a 24-hr recovery period, newts were offered to snakes. The quantity of TTX in the skin sample was assayed in the same manner as TTX in snake tissues as described below.

One snake from the Benton Co., OR, population was encountered in the field on 14 October 2000 after consuming an adult female newt containing eggs. Hanifin et al. (2003) found that the quantity of TTX per clutch of eggs was as much as 1–3 μg, while females contained 0.046–0.487 mg TTX/cm^2 skin. Females were also found to be slightly more toxic than males (Hanifin et al., 2002). The newt ingested by the wild-caught snake was palpated out upon capture in the field; we estimated the newt was consumed 1–2 d previously. The skin was digested, however, the body form was mostly intact, as were the eggs. This opportunity provided an empirical test of TTX retention in the wild. Four days later on 18 October 2000 the snake was euthanized and tissues were collected.

Extraction of Toxin. TTX was extracted from the tissues by modifying the procedure of Hanifin et al. (1999). Two replicates of 125 mg of snake tissue per ml of 0.050 N acetic acid were mixed in a 1 ml Dounce glass homogenizer (Wheaton, USA). Newt tissues were similarly extracted for a final concentration of 10 mg/ml. Extracts were immersed in boiling water for 5 min and cooled in an ice bath before centrifugation at 13,000 rpm for 15 min. The supernatant was collected and filtered though 5,000 NMWL (snakes), or 10,000 NMWL (newts) 0.5 ml Ultrafree-MC Millipore tubes in the centrifuge at 13,000 rpm for 20 min; filtrates were stored at -80°C.

Detection and Quantification of Toxin. Twenty microliters aliquots of sample extracts were quantified on a reverse phase high performance liquid chromatography (HPLC) system by modifying the procedures of Yotsu et al. (1989), and Hanifin et al. (1999). Modification of the HPLC parameters, particularly with respect to the mobile phase, resulted in appropriate phase shifts of elutants such that we were able to quantify accurately TTX in snake tissues. TTX from newt tissues was eluted with an isocratic gradient of 3.0% (2.0% for snakes) by volume acetonitrile, 0.010% (0.013% for snakes) by weight heptafluorobutyric acid, and 0.049 N (0.010 N for snakes) acetic acid. The pH of this mobile phase was adjusted with 50% NH_4OH to pH 5.0 for newts and pH 6.0 for snakes. Analytes were separated with a C18 reverse phase column, Develosil ODS-UG-5 (250 $\times$ 4.6 mm Nomura Chemical, Japan) for newts and Synergi 4 μ Hydro-RP 80A (250 $\times$ 4.6 mm, Phenomenex, USA) for snakes. A flow rate of 0.5 ml per min was produced by a Shimadzu LC-10ADVP pump. The post-column reaction was induced with 5 N sodium hydroxide in a Pickering CRX 400 post-column reactor at 130°C with a flow rate of 0.9 ml per min from a Beckman Model 110A pump. Products were cooled in a water jacket before detection by a Dynamax fluorescence detector Model FL-2 with excitation wavelength set at 365 nm and emission wavelength at 510 nm. An HP 3390A integrator was used to record the chromatographs and calculate peak areas.

TTX peaks in tissue samples were identified and quantified by comparison with a standard of 50 ng TTX (Calbiochem) in 20 μl of 0.050 N acetic acid. Identification of TTX in snake tissues was also evaluated by spiking snake tissues with alleged TTX peaks with TTX standard to elucidate possible co-elution discrepancies. The samples were also compared with TTX-free tissues of two snakes from Bear Lake Co., ID where *T. sirtalis* are not resistant to TTX and *Taricha* are absent. A standard curve for TTX was created between 10 and 500 ng; however, quantification was less accurate at low concentrations in control snake tissues spiked with TTX. Thus, control tissue samples from Bear Lake snakes were spiked with 10, 25, 50, and 160 ng to determine sensitivity of quantification in actual snake tissues. Detector response was linear, accurate, and precise (within 1–10%) at all TTX concentrations in newt tissues and at 50 ng and above in snake tissues. Thus, all snake tissue samples with detectable TTX, but less than 50 ng, were spiked with 47 ng TTX. A snake liver with over 50 ng TTX was spiked with 165 ng TTX as a check on the spiking procedure; quantification in this case was accurate within one SE.

Analysis. For Group A, fed a diet of newts, simple linear regression of the log TTX concentration in the liver vs. time was used to evaluate the assumption that 9 mo was sufficient to purge snakes with a large TTX load. Because the data set did not include points where the concentration of TTX in liver was zero, a 95% confidence interval (CI) for the predicted time that TTX concentration decreased to zero was extrapolated.

Data from treatment snakes (Group D) were analyzed by multiple regression to evaluate the effects of newt toxicity and time (since newt consumption) on snake toxicity. Total newt toxicity was estimated using a formula that incorporates surface area, ratios of general toxin distribution in newts, and the concentration of toxin in dorsal skin by area (Hanifin et al., 2004). Total snake toxicity was conservatively estimated as the concentration of TTX in the liver of each snake multiplied by the mass of the liver. Data were transformed to better approximate normal distributions as follows: log (time), log (newt toxicity + 1), and log (snake toxicity$^{0.8}$ + 1).

In order to put the persistence of TTX in snake livers in context, we estimated the rate of elimination of the toxin based on first order kinetics. For a first order process, the rate of elimination is proportional to the amount of chemical in the body. We assumed a 1st order process because TTX is water soluble and expected to rapidly equilibrate between tissues. Also, a plot of the log concentration vs. time appears to yield a straight line. Based on these assumptions, the decay of TTX over time should follow a one compartment open model. In this case, the decay can be represented by this equation:

$$\log(C/D) = \log C_0 - (k_{\text{el}} \cdot t)/2.303$$

(modified from Medinsky and Klaassen, 1996)

where C represents the concentration of TTX in a snake's liver, D is the dose that an individual snake received (total newt toxicity/mass snake), C_0 represents the y-intercept, t is time, and k_{el} is the first order elimination rate constant. Once the rate of elimination (k_{el}) was estimated, the half-life of TTX in the liver was estimated with the following relationship: $t_{1/2} = 0.693/\, k_{\text{el}}$ (Medinsky and Klaassen, 1996). All data analyses were performed using SAS software, Version 8 of the SAS System for Windows (SAS, 1999).

RESULTS

Because of their larger size, female snakes from the population in Benton Co., OR, were more often able to consume adult newts. Only one male snake consumed a small recently metamorphosed newt during preliminary trials. After garter snakes consumed toxic newts, peaks in the HPLC chromatographs of tissue extracts appeared to correspond to the newt toxin, TTX. The alleged TTX in snake tissues co-eluted with the TTX standard, further verifying the identity of the compound. Snakes from Bear Lake Co., ID, (Group B), which are allopatric to newts, showed no signs of TTX in any tissues, including liver (Figure 1). Snakes from Benton Co., OR, (Group C) fed exclusively a diet of fish for 9 mo also lacked TTX in their livers. During preliminary investigations, screening of a snake 1 hr after consuming a newt revealed TTX in the kidney, liver, skeletal muscle, blood,

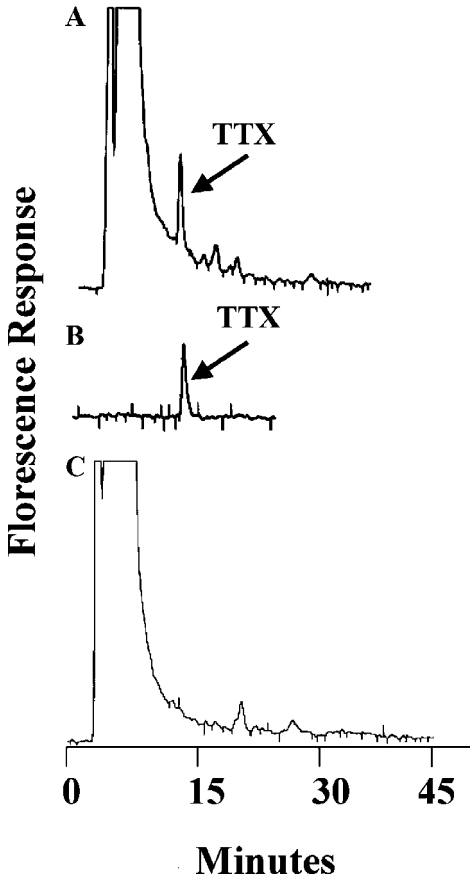

FIG. 1. Chromatographs of (A) a snake liver containing tetrodotoxin (TTX) 1 wk after consuming a newt, (B) commercial TTX standard, and (C) control snake liver from a snake population allopatric to newts.

and heart (by decreasing concentration). In snakes that had consumed a diet of newts (Group A), TTX was present in the liver (up to 7 wk, the last sampling period) and kidney (up to 3 wk), but not in skeletal muscle, cardiac muscle, or blood by 1 wk after newt consumption. One week after consumption of a single newt, TTX was not detectable in kidney, skeletal muscle, cardiac muscle, or blood of treatment snakes (Group D), but was detectable in the liver up to the final sampling period of 4 wk. The liver of the wild-caught snake found with the half-digested newt contained approximately 108 μg of TTX. TTX could not be identified in snake musk with this method because of numerous peaks that eluted

at or near the same time as the toxin. However, the size of peaks in this region indicated that little or no TTX was present in snake musk following newt ingestion. A summary of the location of TTX in snake tissues, number of newts eaten, time elapsed since the last newt eaten, and concentration of TTX in the liver for all snakes is given in Table 1.

Variation between replicate snake tissue extracts fell within the detector variation of 1–10%. Variation between replicate newt tissue extracts was slightly higher at 1–13%, likely due to the small size of tissue samples and sensitivity of the scale used to measure the sample mass. Newts fed to treatment snakes (Group D) ranged in toxicity from 0 to 8.34 mg TTX per newt (mean $\pm$ SD = 3.00 ± 2.19 mg). Tissues collected from one treatment snake (Group D) 4 wk after consuming one newt were excluded from all analyses because this newt contained no TTX. Although TTX was absent from the toxin profile of this newt, other TTX isomers were present.

Snakes in Group A (offered a diet of newts) consumed between four and eight newts in the 5-wk period. Simple linear regression of the log TTX concentration in the liver vs. time for snakes in Group A predicted that our assumption of 9 mo was sufficient time to purge these wild-caught snakes of TTX (Figure 2; df = 7; $F = 7.92$; $P = 0.03$; $r^2 = 0.57$; $y = -0.02x + 1.88$). The regression predicted a loss of toxin in the livers at 62 d with a 95% CI of 24–100 d, approximately one third of the time snakes were held in the lab before being fed newts. Because of the log conversion, a snake with a TTX concentration of zero was excluded, resulting in a more conservative estimate for time required to purge TTX. Because TTX was detected only in the liver of treatment snakes (Group D) 1 wk after consuming a single newt and was not present in other tissues after this time, total snake toxicity was conservatively estimated as the concentration of toxin in the liver multiplied by the mass of the liver. Analyses were then focused on the TTX in the liver of snakes.

We used a partial regression model to examine the effects of total newt toxicity and time on total snake toxicity (Figure 3). There was no interaction between total newt toxicity and time (df = 14; $t = 2.73$; $P = 0.16$), thus, the interaction term was dropped from the model. As expected, total newt toxicity affected the amount of toxin per snake (df = 14; $t = 3.17$; $P = 0.01$). Time was less influential on snake toxicity (df = 14; $t = -1.93$; $P = 0.08$); however, the trend of decreasing toxin concentration over time is evident (Figure 3). For Group D (treatment snakes), the regression of the log of the concentration of TTX in snake liver divided by dose vs. time is shown in Figure 4 (df = 14; $F = 3.97$; $P = 0.07$; $r^2 = 0.234$; $y = -0.03x + 2.0$). Using this regression, the first order elimination rate constant, k_{el} , was calculated at 14.0 d^{-1}. Consequently, an estimate of the half life of TTX in snake liver based on first order kinetics is 9.7 d. After 7 1/2 lives, 99.2% of a chemical is eliminated (Medinsky and Klaassen, 1996), corresponding to 73 d after consumption of a single newt in garter snakes.

TABLE 1. SUMMARY OF ALL SNAKES AND NEWTS, SAMPLING REGIMES, TOXICITIES, AND PRESENCE/ABSENCE OF TETRODOTOXIN (TTX) IN SNAKE TISSUES

Group designation for snake (see text)	Number of newts eaten	Total newt toxicity (mg TTX/newt)	Time since last newt consumption (days)	Tissues in which TTX was detectable (+/−)					Concentration of TTX in snake liver (μg TTX/g liver)	Total snake toxicity (μg TTX)
				Liver	Kidney	Heart	Skeletal Muscle	Blood		
N/A	1	NE	1 hr	+	+	+	+	+	NE	NE
N/A	1	NE	6 d?, field caught	+	NE	NE	NE	NE	17.6	108.0
A	5	NE	8	+	+	−	−	−	48.6	NE
A	4	NE	14	+	+	−	−	−	75.2	NE
A	8	NE	28	+	NE	NE	NE	NE	12.1	NE
A	3	NE	28	+	NE	NE	NE	NE	8.8	NE
A	7	NE	28	+	NE	NE	NE	NE	14.2	NE
A	6	NE	28	+	NE	NE	NE	NE	22.8	NE
A	8	NE	28	+	NE	NE	NE	NE	31.2	NE
A	4	NE	28	−	NE	NE	NE	NE	0.0	NE
A	7	NE	49	+	NE	NE	NE	NE	10.4	NE
B	0	N/A	N/A	−	−	−	−	−	0	0
B	0	N/A	N/A	−	−	−	−	−	0	0
C	0	N/A	N/A, housed in lab 1 year	−	NE	NE	NE	NE	0	0
C	0	N/A	N/A, housed in lab 1 year	−	NE	NE	NE	NE	0	0
C	0	N/A	N/A, housed in lab 1 year	−	NE	NE	NE	NE	0	0
C	0	N/A	N/A, housed in lab 1 year	−	NE	NE	NE	NE	0	0
C	0	N/A	N/A, housed in lab 1 year	−	NE	NE	NE	NE	0	0
C	0	N/A	N/A, housed in lab 1 year	−	NE	NE	NE	NE	0	0
D	1	0	N/A	NE	NE	NE	NE	NE	NE	NE
D	1	2.94	7	+	−	−	−	−	10.6	40.4
D	1	3.09	7	+	−	−	−	−	6.4	21.0
D	1	2.47	7	+	−	−	−	−	25.0	84.3

TABLE 1. CONTINUED

Group designation for snake (see text)	Number of newts eaten	Total newt toxicity (mg TTX/newt)	Time since last newt consumption (days)	Tissues in which TTX was detectable (+/−)					Concentration of TTX in snake liver (μg liver)	Total snake toxicity (μg TTX)
				Liver	Kidney	Heart	Skeletal Muscle	Blood		
D	1	1.53	7	+	−	−	−	−	4.7	20.1
D	1	4.07	7	+	−	−	−	−	19.8	62.1
D	1	2.92	14	+	NE	NE	NE	NE	3.8	12.9
D	1	8.34	14	+	NE	NE	NE	NE	12.3	62.7
D	1	0.59	14	+	NE	NE	NE	NE	2.0	9.2
D	1	1.78	14	+	NE	NE	NE	NE	4.2	15.5
D	1	1.12	21	+	NE	NE	NE	NE	11.5	35.0
D	1	5.88	21	+	NE	NE	NE	NE	10.9	48.7
D	1	4.19	21	+	NE	NE	NE	NE	8.5	35.9
D	1	2.60	21	+	NE	NE	NE	NE	20.1	47.4
D	1	1.03	28	−	NE	NE	NE	NE	0.0	0.0
D	1	5.45	30	+	NE	NE	NE	NE	9.9	32.8

Note. NE = Not examined, N/A = Not applicable.

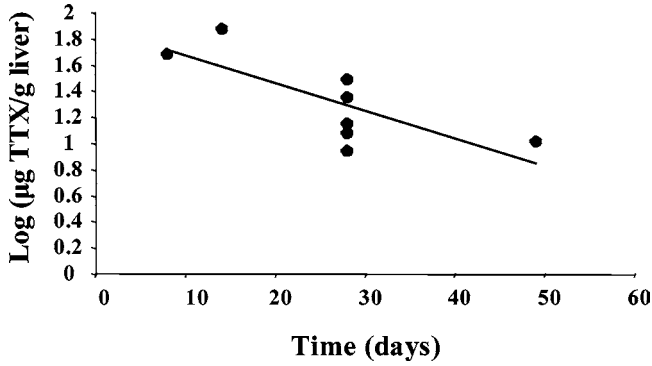

FIG. 2. Logarithmic regression of tetrodotoxin concentration in the liver of snakes fed a diet of newts (Group A) vs. time since last newt consumed. Expanded sampling at 4 wk illustrates variation in snake toxicity.

DISCUSSION

Individuals of *T. sirtalis* from the population in the Willamette Valley, Benton Co., OR, are capable of ingesting massive amounts of the potent neurotoxin TTX. The toxin lingered in snake livers for at least 7 wk and kidneys up to 3 wk after snakes were fed a diet of newts. The presence of TTX in the liver and kidney

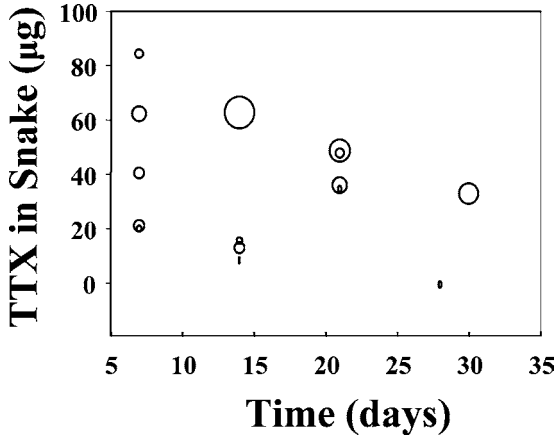

FIG. 3. Relationship of total snake toxicity with time after consumption of one newt (Group D). Bubble area varies in proportion to individual newt toxicity such that larger bubble areas correspond to higher newt toxicity.

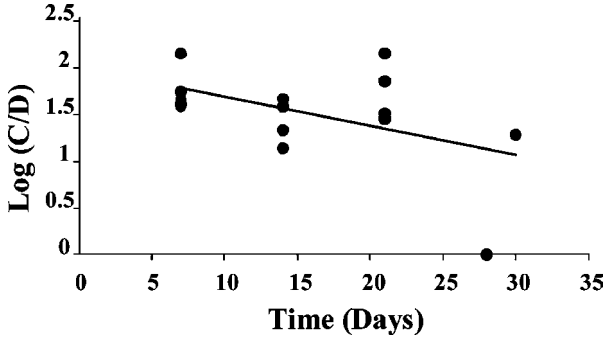

FIG. 4. The regression of the log of the concentration of TTX in snake liver (ng TTX/μl extract) divided by dose (newt toxicity per mass snake in mg TTX/g snake) vs. time (days) for Group D (treatment snakes).

and the absence thereof in other tissues is not surprising. The liver is primarily responsible for detoxification in the body, and Ogura (1958) found that after a subcutaneous injection, large amounts of TTX were present in the kidney of rats, pending elimination. In snakes that consumed only one newt, TTX was present in the liver in appreciable quantities after 1 mo. As expected, snake toxicity depended on the amount of toxin ingested (total newt toxicity). The decrease of toxin concentration over time is also evident, though weak (Figure 3). The shallow slope of toxin elimination over time indicates that TTX is being eliminated at a fairly slow rate.

The estimate of the half-life of TTX in snake liver based on first order kinetics is 9.7 d. The half-life of TTX in rat liver is only 3–4 hr (Ogura, 1958). After 7 1/2 lives, 99.2% of a chemical is eliminated (Medinsky and Klaassen, 1996), thus, rats functionally have no TTX left in the liver after, at most, 28 hr, compared with 73 d for *T. sirtalis* after the consumption of one newt. The persistence of saxitoxin (STX) for 2 mo in the hepatopancreas of lobsters (Cembella and Desbiens, 1994) suggests that there may be differences in longevity of these toxins resulting from divergent physiological characteristics in endotherms and ectotherms. Another possibility is that livers of snakes may contain some kind of molecule that binds TTX. The liver of a species of puffer fish contained a high molecular weight component that released TTX upon digestion with RNase (Kodama et al., 1983). Nagashima et al. (2003) found that puffer fish liver, as opposed to three other fish, differentially accumulated TTX when incubated in a TTX solution and retained the toxin even after incubation in a TTX free solution for an additional 48 hr. TTX is concentrated in the liver of snakes, removed from the sites of action of the toxin. Thus, snakes may not need to expel TTX before they can function normally, resulting in the persistence in livers for several weeks.

This persistence has potential consequences for predators of the snake. Although many snakes (e.g., vipers) are venomous (differentiated by an active delivery of toxin, as in biting or stinging), *Rhabdophis* is the only documented (Akizawa et al., 1985) poisonous snake (passive delivery of toxin, usually by ingestion). Known garter snake predators include herons (*Ardea, Butorides*; Hancock and Kushlan, 1984), ravens and crows (*Corvus*; Shine et al., 2001), hawks (*Buteo*; Fitch, 1965; Richardson et al., 2001), raccoons (*Procyon*), minks (*Mustela*), foxes (*Vulpes*), and badgers (*Taxidea*; Fitch, 1965; and see Rossman et al., 1996). American Bitterns (*Botaurus*; Rapp, 1954; Hancock and Kushlan, 1984) are known to eat *Thamnophis* up to 2 ft in length. Susceptibility to TTX of a variety of taxa has been explored by several investigators (Ishihara, 1918; Kao, 1966; Brodie, 1968). Unfortunately, the variability in newt toxicity between and within populations was not documented until three decades later (Hanifin et al., 1999). Additionally, the standard measurement of TTX quantity is based on mouse units—the reaction of mice to an intraperitoneal injection of toxin extract (Kawabata, 1978). How much purified TTX a mouse unit represents varied between early studies (see Kao, 1966). Hence, the exact amount of TTX administered in these previous studies testing toxin susceptibility is unknown. Yet, predicting the qualitative effect of consuming snake livers on some predators is still possible.

Three weeks after consuming just one newt, the mean quantity of toxin in snake livers was 42 μg ($N = 4$). Birds (common snake predators) are more susceptible to TTX than mammals and reptiles (Ishihara, 1918; Kao, 1966; Brodie, 1968). Several documented instances of predation by birds on *Taricha* yielded no survivors (see Mobley and Stidham, 2000). Although an estimate for a minimum lethal oral dose for birds does not exist, there was some precedent to assume that such a dose could be predicted (Williams et al., 2002) from the minimum lethal dose of subcutaneous injection of TTX for a pigeon (2.0 μg/kg; Ishihara, 1918; Kao, 1966). In mice, approximately 23 times the minimum lethal subcutaneous dose was required to produce a minimum lethal oral dose of TTX (Kawasaki et al., 1973). An estimate of the minimum lethal oral dose estimate for a pigeon is then $23 \times 2.0 = 46$ μg/kg. Even with the inherent variability of such an estimate, the extreme susceptibility of birds to TTX is apparent.

The amount of TTX in snake livers 3 wk after ingesting one newt averaged 42 μg. One may infer that avian predators of garter snakes such as Northern Harriers (420 g), Red-tailed hawks (1080 g), American Bitterns (700 g), and American Crows (450 g; Sibley, 2000) would be severely affected—if not killed—by consuming a snake weeks after the snake had consumed a single newt. Less severe effects of this dose on mammals would be expected. Ten genera of mammals were found to be similarly susceptible to TTX as mice by weight (Brodie, 1968); the oral LD_{50} of TTX for a mouse is 334 μg/kg (Mosher et al., 1964; Kawasaki et al., 1973). Mammalian predators of *T. sirtalis* range in average size from 1–12 kg (Nowak, 1991), thus, a TTX dose of the order of 42 μg would not

likely be lethal. However, because TTX may also cause an emetic response and the onset of symptoms is extremely rapid (Kao, 1966; Brodie, 1968), mammalian predators may be negatively affected at this dose.

These comparisons have been made with the apparent toxicity of snakes based on the consumption of a single newt. A snake that ate seven newts during a 5-wk period possessed considerable amounts of TTX in the liver 7 wk after this snake had been switched to a diet of fish—about 60 μg. The number of newts a snake may eat is unknown; however, one snake from Benton Co., OR, consumed eight adult newts in 2 wk in the lab (Brodie, 1968). The snake discovered in October with a half-digested newt in its stomach contained approximately 108 μg TTX. Tissues were collected from this snake 4 d after the newt had been palpated out of the stomach. Had the snake been able to completely digest the newt, the concentration of TTX in the liver may have been higher. Regardless, the occurrence of large quantities of TTX in wild snakes was verified.

The literature, combined with our data, suggests that snakes may harbor TTX in the approximate range, or just below a lethal dose for certain predators. Shine et al. (2001) found that crows were the main predators of *T. sirtalis* emerging from dens in Manitoba (outside the geographic range of *T. granulosa*). Remarkably, the crows excised the garter snake livers preferentially (an easily located, highly nutritional source in snakes), and consumed several in quick succession. Notably, live snakes were observed with ventral scars that have been attributed to partial liver removal by attacks from bird predators (M. Pfrender and R. Mason, pers. comm.).

If a predator became sick after ingesting a toxic snake liver but survived, it might learn to avoid these snakes. The ability of birds to acquire aversions to aposematically colored prey has been demonstrated repeatedly (Nicolaus et al., 1983; Roper and Wistow, 1986), even after just one exposure to noxious food items (Brower et al., 1970). The possibility of predators learning to avoid poisonous garter snakes may be accentuated (Fisher, 1930) by the fact that TTX is fast-acting, may cause an emetic response (Kao, 1966; Brodie, 1968), and garter snakes may exhibit defensive displays towards predators including open mouth strikes and flattening of the body to appear larger (Arnold and Bennett, 1984). Snakes in Manitoba reveal their distinctive red lateral coloration during the body flattening displays in response to a crow model (Shine et al., 2000). *T. sirtalis* from Benton, Co., OR, have far more brilliant red coloration, not only in their distinct lateral bars, but also on their head, which contrasts with their black background. Contrasting prey coloration accelerates predator learning (Schuler and Hesse, 1985; Sillén-Tullberg, 1985). Avian predators are visually oriented, discriminate objects based on color (Cuthill and Bennett, 1993; Hunt et al., 1997), and their foraging behavior can be influenced by variation in color and light (Maddocks et al., 2001). The contrasting colored rings of venomous coral snakes (*Micrurus, Micruroides*) and their sympatric nonvenomous kingsnake and milk snake mimics (*Lampropeltis*)

are aposematic to avian predators (Brodie and Janzen, 1995; Pfennig et al., 2001). Additionally, Hensel and Brodie (1976) found that Blue Jays (*Cyanocitta cirstata*), Common grackles (*Quiscalus quiscula*), and Brown Thrashers (*Toxostoma rufum*) recognized not only red on black warning coloration, but minute variations in form of distasteful salamander prey.

A combination of cues, such as chemosensory (Terrick et al., 1995), combined with aposematic colors may accelerate predator learning. Garter snakes often void their cloaca when threatened by predators, and the odor of the musk they secrete is distinctive. Musk, as an additional cue, may facilitate predator learning.

Additionally, Roper and Redston (1987) found that the rate of acquisition and duration of learned avoidance for predators may be increased by the frequency of exposure to aversive-tasting prey. The probability of encountering a toxic snake does not necessarily equate with the frequency of toxic snakes in the population. Brodie and Brodie (1999a) have shown that highly resistant individuals of *T. sirtalis* have reduced crawl speed in comparison with non-resistant individuals from the same population. Therefore, the ability of resistant snakes to escape from their predators may be reduced even before consuming a newt. As resistance increases, snakes are more likely to successfully consume a newt (Williams et al., 2003). After consuming a newt, the mobility of a snake is further impaired by the effects of TTX for up to several hours (Brodie and Brodie, 1990; Williams et al., 2003). During this time, snakes are especially vulnerable to predators. Finally, size matters—female snakes, which are larger than males, are more often able to consume adult newts. The combination of lethargy due to intoxication, bright coloration, visual sensitivity of birds, and larger size would render poisonous snakes more conspicuous. Conspicuousness increases the rate of consumption of a prey item, at least initially (Gittleman et al., 1980; Gittleman and Harvey, 1980). A higher probability of encountering toxic prey results in predators experiencing a higher proportion of unpalatable prey, which may accelerate predator learning. The higher proportion of toxic prey encountered also increases the possibility of an automimetic complex occurring within a population (Brower et al., 1970). Additionally, as the probability of prey entering the perceptual field of a predator increases, selective pressure on antipredator mechanisms, including toxicity, increases (Brodie et al., 1991). Because toxic snakes should be more frequently encountered than their innocuous counterparts, the evaluation of the role of snake toxicity as an antipredator mechanism requires consideration.

The occurrence of TTX in livers of garter snakes in such large quantities effectively renders some snakes of this population poisonous to potential predators. How poisonous depends on individual variation in newt toxicity and consequent variation in toxicity of snakes as well as specific and individual differences in predator susceptibility to TTX, which remains to be tested empirically. Whether toxicity in snakes constitutes chemical protection, or otherwise benefits the snakes is unknown and depends on the probability of predators encountering a poisonous

snake and the predators' ability to learn to avoid them. However, individual snakes that consume even a single newt are subsequently poisonous at least 3–4 wk later. The persistence of foreign toxins in an organism may be the necessary first step for evolution of the sequestration of toxin for consequent protection. The possibility that some populations of garter snakes may employ TTX obtained from their newt prey as an antipredator mechanism adds yet another dimension to the multifaceted coevolutionary interactions between *T. granulosa* and *T. sirtalis*.

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INTERSPECIFIC DIFFERENCES IN *AETHIA* SPP. AUKLET ODORANTS AND EVIDENCE FOR CHEMICAL DEFENSE AGAINST ECTOPARASITES

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Abstract—The true auklets (*Genus Aethia*) are small planktivorous seabirds of the Bering Sea and North Pacific. Two species, the crested and whiskered auklets produce volatile citrus-like odorants. We here show that the whiskered auklet odorant is composed predominantly of two odd-numbered aldehydes (heptanal and nonanal) with no detectable unsaturated aldehydes. By comparison the crested auklet odorant is dominated by even-numbered aldehydes, both saturated and monounsaturated, ranging in size from 6 to 12 carbons. This is evidence of species-specific acquisition or biosynthetic pathways. We clarify the chemistry of the crested auklet odorant. We cite evidence that the C-12:1 aldehyde in crested auklets is actually two isomers, (*Z*)-4-dodecenal and (*Z*)-6-dodecenal. We also report on experimental evidence that aldehyde constituents kill and repel ectoparasites. Efficacy of the aldehydes may increase when they are combined in a mixture. The repellency of the mixture increases with chemical concentration. This suggests that individuals with higher chemical production are likely to repel ectoparasites more effectively.

Key Words—Crested auklet, whiskered auklet, aldehydes, (*Z*)-4-dodecenal, (*Z*)-6-dodecenal, synthesis, chemical defense, ectoparasites, pheromones, seabirds.

INTRODUCTION

Aethia auklets are some of the most locally abundant avifauna in the Bering Sea, with colony sizes ranging up to 10^7 individuals (Springer et al., 1993). They dive

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TABLE 1. PERCENT COMPOSITION OF ODORANT IN CRESTED AUKLETS (*A. cristatella*) COMPARED TO WHISKERED AUKLET (*A. pygmaea*) AS DETERMINED FROM FEATHER EXTRACTS

Compounds	*A. cristatella*	*A. pygmaea*
Hexanal	21.4	—
Hexanoic acid	7.7	—
Heptanal	—	22.3
Octanal	40.7	—
Octanoic acid	3.5	—
Nonanal	—	22.3
Decanal	3.4	9.7
Z-4-Decenal	8.2	—
Z-4-Dodecenal	7.5	—
Z-6-Dodecenal	7.5	—
Hexadecanol	—	45.6

at sea for their prey, and three of the four species feed almost exclusively upon zooplankton. Of these, the crested and whiskered auklet (*Aethia cristatella and pygmaea*) have an unusual citrus-like odor (Humphrey, 1958; Jones, 1993), while the least auklet (*A. pusilla*) does not have a noticeable plumage odor. The parakeet auklet (*A. psittacula*) has broad diet preferences that include invertebrates and fish, but a bill morphology that is probably best adapted to feeding upon gelatinous zooplankton, e.g., jellyfish (Harrison, 1990; Jones et al., 2001). The parakeet auklet also lacks the "citrus" odorants (Jones et al., 2001; Hagelin et al., 2003). The phylogenetic relationships of the *Aethia* auklets have not been resolved using either morphological or molecular characters (Friesen et al., 1996). However, it seems plausible that crested and whiskered auklets are the most closely related due to similarities in their plumage ornaments, odorants, and vocalizations (Gaston and Jones, 1998).

In a preliminary report, we described the major components of the "citrus" odor of the crested auklet as a series of volatile aldehydes (Douglas et al., 2001, Table 1). These are dominated by even-numbered, saturated and monounsaturated compounds, 6-12 carbons in length (hexanal, octanal, decanal, (*Z*)-4 decenal, and an unidentified C-12:1 aldehyde).

We previously proposed that the odorant might be an avian chemical defense and a signal of mate quality (Douglas et al., 2001, 2002). Two major constitutents of the crested auklet's odorant, *n*-octanal and *n*-hexanal, are secreted in the metapleural glands of stinkbugs and are potent invertebrate repellents (Aldrich, 1988). This suggests a convergence upon a common solution in defensive chemistry against invertebrate enemies (Douglas et al., 2001). Predation is the apparent selection pressure acting upon Heteropteran insects, whereas parasitism is the hypothetical selection pressure acting upon auklets.

Indeed, parasitism appears to motivate exogenous anointment in many avian species. Many birds rub their plumage with materials that have chemical properties similar to the crested auklet's odorant. For example, birds crush ants in their bills and rub the secretions of ant metapleural glands through their plumage, a behavior known as "anting" (Ehrlich et al., 1986; Clayton and Vernon 1993). Similarly, the addition of green plant materials to bird nests has been discussed as a method of chemical defense against ectoparasites (Clark and Mason, 1985, 1988; Gwinner et al., 2000). Experimental evidence for the defensive efficacy of these behaviors remains elusive (Hart, 1997; Gwinner et al., 2000).

If the exogenous application of defensive chemicals is enigmatic, the endogenous production of chemical defenses is virtually unexplored. The pitohuis and *Ifrita kowaldi* of New Guinea are the few known examples (Dumbacher et al., 1992, 2000). These species produce or sequester batrachotoxins. These are potent nerve toxins with potentially broad defensive targets including both predators and avian lice (Dumbacher, 1999). Pitohuis also produce a sour odor that may signal their toxic properties (Dumbacher et al., 1992). Interestingly, odoriferous species are reported from eighty avian genera worldwide (Weldon and Rappole, 1997).

We herein clarify the chemistry of the crested auklet odorant, verify that its constituents repel or kill relevant ectoparasites in a dose-dependent fashion, and compare the odorants of crested and whiskered auklets.

METHODS AND MATERIALS

Birds. Crested Auklets. Six specimens were sacrificed and collected at Kiska I. (Lat. 52°06′N, Long. 177°36′E), Aleutian Islands, Ak on 26 May 2000 and frozen soon afterwards. These specimens were shipped frozen to our chemical laboratories at Virginia Military Institute and stored in an −80°C freezer. Analyses of odor components were conducted by headspace analysis and by extraction of the neck feathers. In the first method, volatiles were collected from two specimens as each thawed in a 2000 ml glass beaker covered with aluminum foil. Headspace analysis was conducted using a solid phase microextraction (SPME) fiber (1 cm in length) coated with 65 μm of polydimethyl siloxane/divinylbenzene partially crosslinked (Supelco Corp.). The fiber was positioned near the speciment's neck, and samples were collected for 4.5 hr and subsequently subjected to GC/MS analysis. For extraction, feathers clipped from the nape of three frozen birds were gently macerated and extracted in 1 ml of either methylene chloride or methanol for 2 min. The extract was analyzed by GC/MS. Subsequent analyses have shown that samples obtained from live birds with methanol feather extraction and headspace collection yield similar results.

Whiskered Auklets. Six specimens were sacrificed and collected near Tanager Pt., Chugul I. (Lat. 51°55′N, Long. 175°53′ W), Aleutian Island, AK on 4 June 2001 and frozen soon afterwards. These specimens were shipped frozen to the University of Alaska Fairbanks and stored in a −20°C freezer. The nape feathers from six specimens were gently macerated and extracted in 1 ml of methanol for 2 min. The extracts were analyzed by GC/MS.

Chemical Analysis. Gas chromatography–mass spectrometry was carried out in the EI mode using a Shimadzu QP-5000 GC/MS equipped with a RTX-5, 30 m × 0.032 mm i.d. column. The instrument was programmed from 60 to 250°C at 10°/min. Vapor phase FT-IR spectra were obtained using an Hewlett-Packard model 5965B detector interfaced with an Hewlett-Packard 5890 gas chromatograph fitted with a 30 m × 0.25 mm RTX-5 Amine column. High Resolution mass spectrometry was performed on a JEOL SX102 instrument. Identification of the compounds was confirmed by direct comparison of their mass spectra and retention times with those of commercial or synthetic pure compounds.

Synthesis. It was necessary to synthesize standards to confirm identities of the 12-carbon aldehydes in the crested auklet odorant because no commercial standards are available.

(*Z*)-4-Dodecenal. A solution of sodium methylsulfinyl–methanide (16 mmol) was prepared from 400 mg of NaH in 5 ml of dimethylsulfoxide (DMSO) and combined with 15 ml of tetrahydrofuran (THF). Triphenyl-phosphonium bromide, prepared from 2-(3-bromopropyl)-1,3-dioxolane and triphenylphosphine, in the quantity of 3.6 g (7.9 mmol) was added to this mixture at 25°C under argon. After 10 min, the mixture was cooled to 0°C and treated with 1.2 ml of freshly distilled octanal. The mixture was stirred for an hour, poured into distilled water, and extracted with ether three times. The ether fraction was dried under reduced pressure, and the residue was extracted several times with petroleum ether. After removal of the solvent, the mixture was heated to 80°C for 2 hr in 5 ml of concentrated acetic acid. The solvent was removed, and the residue was resuspended in ether, and neutralized with saturated $NaHCO_3$. After drying over $MgSO_4$, removal of the solvent provided 1.2 g of a mixture that was greater than 80% a single component, MS m/z (rel %) 164 (M-18, 2), 138(5), 111(3), 110(4), 109(3), 98(10), 97(12), 96(7), 84(72), 83(32), 82(10), 81(12), 79(10), 70(14), 69(15), 68(12), 67(23), 57(10), 56(13), 55(48), 54(30), 53(5), 43(67), 41(100). A sample of this product was suspended in petroleum ether and heated to 60°C with dimethylhydrazine for 4 hr. After the solvent was removed, the (*Z*)-4-dodecenal was obtained as a single major component, MS m/z (rel %) 224(M-18, 3), 209(1), 182(3), 180(1), 156(1), 139(2), 125(2), 111(1), 95(2), 86(9), 85(100), 69(3), 67(5), 55(7), 44(36).

(*Z*)-6-Dodecenal. A solution of sodium methylsulfinylmethanide was prepared from 600 mg of NaH in 15 ml of DMSO, and this was placed in 15 ml of THF. To this was added a solution containing 4.4 g (10 mmol) of triphenylphosphonium (6-hydroxyhexyl) bromide dissolved in 12 ml of DMSO under literature

conditions (Horiike et al., 1978). After 20 min, 1 ml of freshly distilled hexanal was added, and the reaction was worked up in the prescribed manner. Half of the crude product was added to a slurry of pyridinium chlorochromate in CH_2Cl_2 (3 g/50 ml). This mixture was stirred for 3 hr. After the addition of 150 ml of ether, the mixture was filtered through a short florisil column. The solvent was removed to provide 0.5 g of an oil that was 80% a single component: MS *m*/*z* (rel %) 164 (M-18, 3), 135(2), 121(7), 98(12), 97(16), 96(9), 95(9), 94(5), 93(10), 84(16), 83(20), 82(18), 81(25), 80(9), 79(24), 70(24), 69(23), 68(10), 67(34), 57(22), 56(20), 55(84), 54(40), 53(10), 43(44), 41(100). A sample of this was suspended in petroleum ether and heated to 60°C with dimethylhydrazine for 4 hr. After the solvent was removed, the (*Z*)-6-dodecenal was obtained as a single major component: MS *m*/*z* (rel %) 224 (M-18, 3), 209(1), 195(1), 180(6), 167(1), 153(2), 142(9), 122(9), 99(8), 95(8), 86(40), 85(27), 73(15), 67(11), 60(30), 59(48), 45(42), 44(100), 43(50), 42(45), 41(49); HRMS, calculated for $C_{14}H_{28}N_2$: 224.2252: observed 224.2247.

Repellency Trials. We adopted a moving object bioassay for tick repellency tests from Dautel et al. (1999). A heated rotating drum emulated host cues of heat and motion. The drum (21.3 cm long × 11.5 cm diam.) full of water was maintained at 38°C with an aquarium heater (Slim-Tech #8814PC). A Pioneer turntable powered by a Dayton DC Motor (Model 1Z835) and regulated by a DC pulse-width modulated speed controller (Model CK 1400, Carl's Electronics, Sterling, MA) rotated the drum at 10.5 rpm. A raised plate (7.6 × 3.2 × 0.2 cm thick) on the drum's surface afforded a point of attachment where questing nymphs could attach to the artificial host per their natural behavior. These nymphs were placed on a metal rod (4 mm diam.), positioned perpendicular to the rotating drum with the end of the rod just 1.1 mm from the surface of the plate (after Dautel et al., 1999). The rod was sheathed in heat shrink tubing to improve the tick's ability to adhere. Filter paper (Whatman 2 Qualitative) was cut to fit the raised plate; chemicals were added to the filter paper until saturated (0.008–0.025 ml/cm^2). The paper was allowed to volatize 10 min prior to placing it on the raised plate. Volatile repellents wane in effectiveness after 1 hr (e.g., DEET; Dautel et al., 1999). If a trial extended more than 1 hr, the filter paper was saturated again, allowed to volatize for 10 min, and then the trial was resumed.

During laboratory and field trials, individual nymphs were picked up with a fine-haired camel brush and placed 8 mm from the rod tip, oriented towards the rotating drum. Each tick was observed for 2 min, and the following data were recorded: approached drum (yes/no), time to walk to rod tip, time at tip, attached to drum (yes/no), and druation of attachment. We used nymphs of *Amblyomma americanum* for laboratory trials, and nymphs of *Ixodes uriae*, (Acari: Ixodidae) for field trials. *Ixodes* ticks may occur in high densities at some colonies, and they parasitize auklets throughout most of their range. *Amblyomma* ticks do not occur in the auklets' range; they do commonly parasitize birds and mammals. Nymphs

of *Amblyomma* were obtained from Oklahoma State University. *Ixodes* ticks for field trials were obtained from a crested auklet colony above Yukon Harbor, Big Koniuji Island, AK. These *Ixodes* ticks were deposited in the U.S. National Tick Collection (curated at Georgia Southern University) under accession number RML 123386.

The repellency trials addressed two hypotheses: (1) Do the dominant odorant constituents of crested auklets repel ticks in a dose dependent fashion? (2) Do different aldehyde constituents of crested auklets differ in their level of repellency? In order to answer these questions, we compared duration of attachment in two lab experiments (I and II) and a field experiment (III). The objective of Experiment I was to determine whether octanal, the chief ingredient in the crested auklet's odorant, is repellent to ticks. This research was conducted in the lab with a treatment of 10% octanal in ethanol, an ethanol control, and a blank control. We also tested the ethanol control to determine whether it is more repellent than the blank filter paper. In Experiment II, the objective was to calculate a dose–response curve for repellency and determine the level of significant effect. A synthetic cocktail of the crested auklet odorant constituents (40% octanal, 21% hexanal, 8% (*Z*)-4 decenal, 3% decanal, 7% hexanoic acid, 3% octanoic acid) was presented at three concentrations (100%, 10% in ethanol, and 1% in ethanol). The percentages of aldehydes refer to relative abundance of each aldehyde per total volume of cocktail (the actual purity of each aldehyde in the auklet odorant is relatively high, e.g., >92% in the case of octanal; Douglas et al., 2001). These treatments were compared to an ethanol control. Dodecenals were not used in the cocktail because they are not commercially available. Experiment III was conducted at Big Koniuji Island in the Shumagin Islands, AK with three treatments: (1) octanal, (2) decanal, (3) aldehyde mixture (octanal 40%, hexanal 20%, decanal 3%. in ethanol). These treatments were compared to an ethanol control. The objective of was to compare the repellency effect of crested auklet odorant constituents individually and in combination.

We used the following synthetic chemicals for all bioassays: Hexanal 96% (C.A.S.: 66-25-1), Octanal 99% (C.A.S.: 124-13-0), Decanal 95% (C.A.S.: 112-31-2), Hexanoic acid 99+% (C.A.S.: 142-62-1), Octanoic acid 99% (C.A.S.: 124-07-2) by ACROS Organics, and (*Z*)-4 Decenal 95% (C.A.S.: 21662-09-9) by Lancaster Synthesis. Laboratory trials were conducted at 22°C and at moderately high humidity. Field trials were conducted inside a tent with moderately high humidity and at ambient temperatures that fluctuated between 14–22°C. Results of control and experimental trials were compared with an independent measures *t*-test, after evaluating homogeneity of variance.

Mortality Tests. Mortality trials were conducted in the field with nymphs and adults of *Ixodes uriae* at Big Koniuji Island AK. The objective was to determine whether small quantities of a crested auklet odorant constituent would cause mortality. We chose octanal because it is the most abundant. Distilled water was

used for the control. Each tick was placed in an individual glass vial (Fisher #03-339-21F). Six adults and four nymphs were placed in the control. Seven adults and three nymphs were placed in the octanal treatment. We used a 100 μl Hamilton Syringe (Model 84859 810RN) to apply the smallest quantity of liquid possible (5 μl) without touching the syringe needle to the tick.

Mortality trials were also conducted with auklet lice obtained from crested auklets at Little Diomede Island, AK on August 28, 2002, using a carbon dioxide fumigant, according to methods reported in Visnak and Dumbacher (1999). Fifteen lice were used in each of three treatments for a total of 45 lice. In each treatment, we placed an individual louse on an individual crested auklet feather. Next 1 μl of substance (octanal, (*Z*)-4-decenal, or tap water with soap) was applied to the opposite side of the feather with a Hamilton Syringe. This was replicated 15 times for each treatment. Lice were checked at the time of application and then at 1 min, 1, and 5 hr elapsed time after application.

The experimental design was identical in all respects. Feathers for this experiment were slected from a single crested auklet specimen in June at Big Koniuji Island. The feathers were first placed in a petri dish and left in sunlight. Light and exposure to oxygen break down the auklet aldehydes. After 1 week, the remaining concentrations of aldehydes in feathers, if present, are below the threshold of human detection. After 1 month, we selected feathers of identical size, shape, and length; all feathers had similar surface areas. Five feathers were suspended by the calamus (equidistant from each other) from an aluminum foil lid. Next, the foil was firmly attached to the rim of a 500 ml glass jar. Three glass jars were prepared for each treatment, and this accommodated 15 lice for each treatment. We used tap water with soap (1 drop/cup) as a control. Soap was added as a wetting agent since avian feather oils have hydrophobic properties. To conduct the trials we removed the foil lid and lay it upside down with the feathers facing straight up. A louse was placed on each feather. The substance was applied to the feather. Then, the foil lid was secured, and the lice were observed through the jar. These lice were deposited into the frozen collections of the Price Institute for Phthirapteran Research at University of Utah.

RESULTS

Chemical Analysis. Crested Auklets. Solid phase microextraction (SPME) from two birds provided the initial indication that the odor was due to the presence of short chain aldehydes (Douglas et al., 2001). GC/MS analysis of the absorbed volatiles revealed the presence of octanal, (*Z*)-4-decenal, and decanal, all confirmed by comparison with commercial samples (Douglas et al., 2001). Solvent extraction of the neck feathers from three birds provided a more complete and consistent analysis. GC/MS analysis of the extract revealed a complex mixture of high molecular weight oils along with a number of volatile components. The

latter contained hexanal (16–26%), octanal (38–43%), (*Z*)-4-decenal (7.4–8.6%), decanal (3–4%), and a C-12:1 aldehyde (13–18%): MS *m/z* (rel %) 164 (M-18, 2), 138(2), 135(2), 121(4), 98(9), 97(10), 96(5), 95(4), 84(22), 83(18), 82(11), 81(17), 80(7), 79(14), 70(14), 69(18), 68(10), 67(25), 57(20), 58(17), 55(61), 54(25), 43(43), 41(100). Our analyses did not find trace chemicals reported by Hagelin et al. (2003).

Initially, the C-12:1 aldehyde was assumed to be a (*Z*)-4 isomer, homologous with the (*Z*)-4-decenal present in the mixture. Closer inspection of the GC peak showed that the intense ion at $m/z = 84$, characteristic of (*Z*)-4-dodecenal, was not congruent with the total ion current for that peak, matching only the leading edge of the peak. When the peak was scanned from the beginning to the end, the ion at $m/z = 84$ changed in intensity from 72 to 16 relative %. Further examination of the spectra from the trailing edge of the peak suggested the presence of (*Z*)-6-dodecenal (Horiike and Hirano, 1988). These data indicated that the unidentified C-12:1 aldehyde was actually a mixture of two isomers.

Dimethylhydrazones of the extract were prepared, since the mass spectra of these derivatives have been used to assign double bond position in unsaturated aldehydes (Attygalle et al., 1989, 1998). The front edge of the peak for the dimethylhydrazone of the dodecenal ($m/z = 224$, M^+), showed an intense ion at $m/z = 85$ (80%), and the trailing edge of the peak showed an ion at $m/z = 142$ (10%). Selected ion monitoring showed that these two ions were not congruent with each other or with the total ion current for this peak. The intense ion at $m/z = 85$ in these derivatives is indicative of a C-4 double bond, while the ion at $m/z = 142$ results from the splitting out of cyclohexene that occurs in the mass spectra of these derivatives with a double bond at the C-6 position (Figure 1).

The assignment of both (*Z*)-4-dodecenal and (*Z*)-6-dodecenal was confirmed by direct comparison of the natural component with synthetic samples prepared

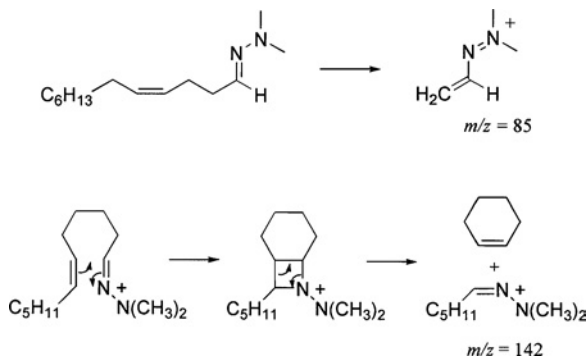

FIG. 1. Fragmentation ions from (*Z*)-4 and (*Z*)-6 dodecenals.

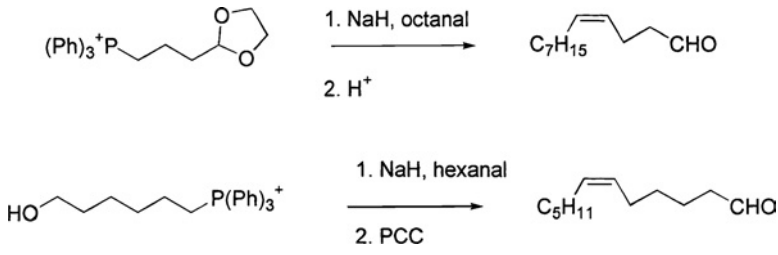

FIG. 2. Preparation of (*Z*)-4 and (*Z*)-6 dodecenals.

as shown in Figure 2. The Wittig conditions used in the syntheses produced small amounts of the E isomers (<5%) sufficient to permit the assignment of double bond geometry by comparison, and the natural material matched the major (Z) synthetic component in both cases. A mixture of the dimethyl-hydrazones from the synthetic (*Z*)-4-dodecenal and (*Z*)-6-dodecenal also had identical mass spectral properties and GC retention times to the chromatographic peak for the dimethylhydrazone of the C-12:1 aldehyde from the natural extract.

Whiskered Auklets. Chemical composition of crested auklet and whiskered auklet odorants are compared in Table 1. Analysis of whiskered auklet feather extracts revealed the presence of heptanal, nonanal, decanal, and hexadecanol in an average ratio of 2.3:2.3:1:4.7. The MS and GC retention times were identical to those of authentic samples.

Repellency Trials. Moving object bioassays demonstrated that the aldehyde odorant of crested auklets is repellent to ticks. Duration of attachment to the artificial host was significantly less for aldehyde treatments than for the ethanol and blank filter paper controls. In all three experiments, the majority of ticks (>93%) consistently walked to the end of the rod and many of these quested for the artificial host. However, they were repelled when the filter paper with aldehydes passed the end of the rod. In these instances, some nymphs returned to quest a second and third time but were repelled on each occasion. Some of those ticks that did attach to aldehyde treated filter paper fell off soon afterwards.

Experiment I. Nymphs of *A. americanum* were repelled by the octanal treatment (Figure. 3). Duration of attachment was greater for the ethanol control (72.4 8.8, sec $N = 30$) than for the octanal treatment (23.3 $\pm$ 11 sec, $N = 20$), $t(47)_{1\text{-tailed}} = 3.50$, $P < 0.001$. Duration of attachment was also greater for the blank paper control (62.7 9.8 sec, $\pm$ $N = 30$) compared to the octanal treatment, $t(48)_{1\text{-tailed}} = 2.33$, $P < 0.05$. Fewer ticks attached to the octanal treatment (25%) than the ethanol control (80%), $t(48)_{1\text{-tailed}} = 4.0$, $P < 0.001$. The difference was also significant in comparison with blank filter paper; 63% of ticks attached to this control, $t(48)_{1\text{-tailed}} = 2.13$, $P < 0.05$. The ethanol control did not differ in duration of attachment from the blank control ($t(58) = 0.73$, $P_{1\text{-tailed}} = 0.24$).

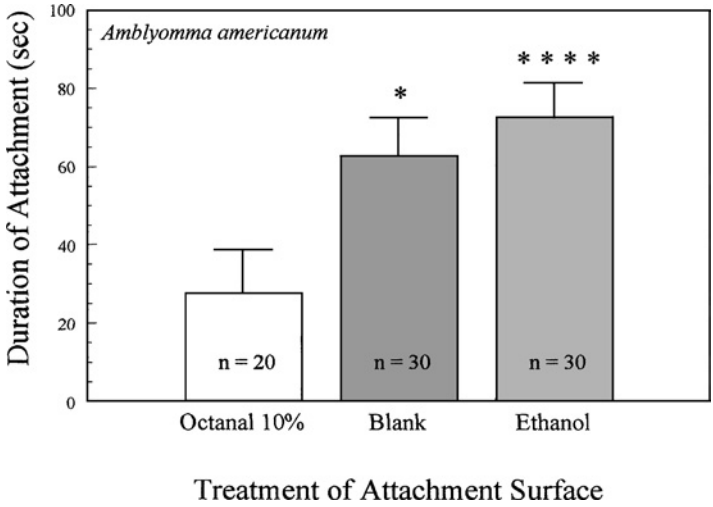

FIG. 3. Experiment I: Ticks were repelled in a moving object bioassay by filter paper trated with octanal. Nymphs of *Amblyomma americanum* were repelled by filter paper treated with 10% octanal in ethanol. Duration of attachment to the artificial host was less for the octanal treatment than for either control treatment (blank filter paper and ethanol treated filter paper). A single asterisk indicates significance at $P < 0.05$. Four asterisks indicate significance at $P < 0.001$.

Ticks were not repelled by ethanol. If anything, ethanol may be a mild attractant. A slightly higher percentage of ticks attached to the ethanol control (80%) compared to the blank control (63%), and this difference bordered on significance ($t(58) = 1.70$, $P_{1\text{-tailed}} = 0.047$).

Experiment II. Repellency of the aldehyde odorant increases with concentration (Figure. 4). The average duration of attachment did not differ between the 1% concentration (42.8 ± 10 sec) and the blank control (49.9 ± 9.7 sec), $t(57)_{1\text{-tailed}} = 0.399$, $p = 0.34$. Increasing the aldehyde concentration to 10% reduced the average duration of tick attachment to 13.5 sec (± 5.9), and this was less than the blank control $t(48)_{1\text{-tailed}} = 3.20$, $P < 0.005$. At 100% concentration, the average duration of attachment was 0.3 sec (± 0.5), significantly less than the blank control, $t(29)_{1\text{-tailed}} = 5.09$, $P < 0.001$.

Experiment III. Constituents of the crested auklet aldehyde odorant were repellent to nymphs of *I. uriae*, an ectoparasite of crested auklets (Figure. 5). Duration of attachment for the ethanol control averaged 63.4 sec (± 8.9) vs. 10.8 sec (± 5.4) for octanal, 31.6 sec (± 7.5) for decanal, and 24.3 sec (± 7.1) for the aldehyde mixture. All of these differences were statistically significant: octanal, $t(58)_{1\text{-tailed}} = 4.85$, $P < 0.01$; decanal, $t(58)_{1\text{-tailed}} = 2.06$, $P < 0.05$;

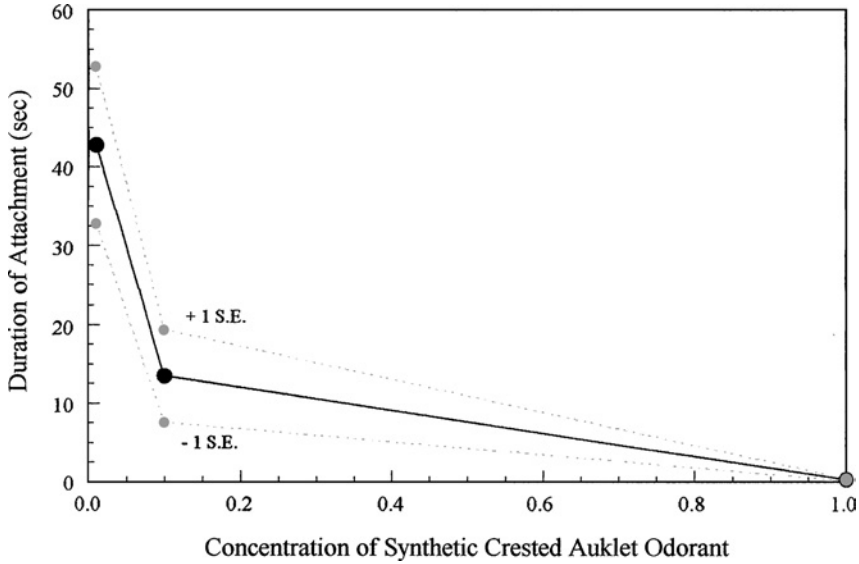

FIG. 4. Experiment II: Nymphs of *Amblyomma americanum* exhibit dose-related response to varying concentrations of synthetic crested auklet odorant in ethanol. Response is measured as duration of attachment (sec) to an artificial host. Repellence is higher at higher concentrations. Attachment time was greater for the control than the synthetic blend at 100% ($P < 0.001$) and 10% ($P < 0.005$) but not at 1% concentration ($P = 0.34$).

aldehyde mixture consisting of 40% octanal, 20% hexanal, 3% decanal in ethanol, $t(58)_{\text{1-tailed}} = 2.78$, $P < 0.01$. Nymphs of *I. uriae* were more likely to attach to the ethanol control than the aldehyde treatments. Seventy percent of nymphs ($N = 30$) attached to the control. By comparison, only 20% of nymphs attached to octanal ($t(58)_{\text{1-tailed}} = -4.43$, $P < 0.001$); 43% attached to decanal ($t(58)_{\text{1-tailed}} = -2.13$, $P < 0.05$); and 40% attached to the aldehyde mixture ($t(58)_{\text{1-tailed}} = -2.41$, $P < 0.01$). Repellency of the dilute aldehyde mixture (63% concentration in ethanol) was equivalent to nearly pure decanal (Figure. 5). Duration of attachment did not differ for the two treatments ($t(58)_{\text{2-tailed}} = 1.67$, $P > 0.1$).

Mortality Trials. Nymphs in the octanal treatment became moribund in less than 15 min, and adult ticks did so in less than 1 hr. None of these ticks recovered within 2 days. All ticks in the control treatment were still active 2 days later. All lice in the experimental treatments (octanal and (*Z*)-4-decenal) became moribund within seconds. All lice in the control treatment (tap water with soap) were active up to 12 hr later. The control and (*Z*)-4-decenal treatments included lice from the genera *Austromenopon* and *Quadraceps*. The octanal treatment only included lice from the genera *Austromenopon*.

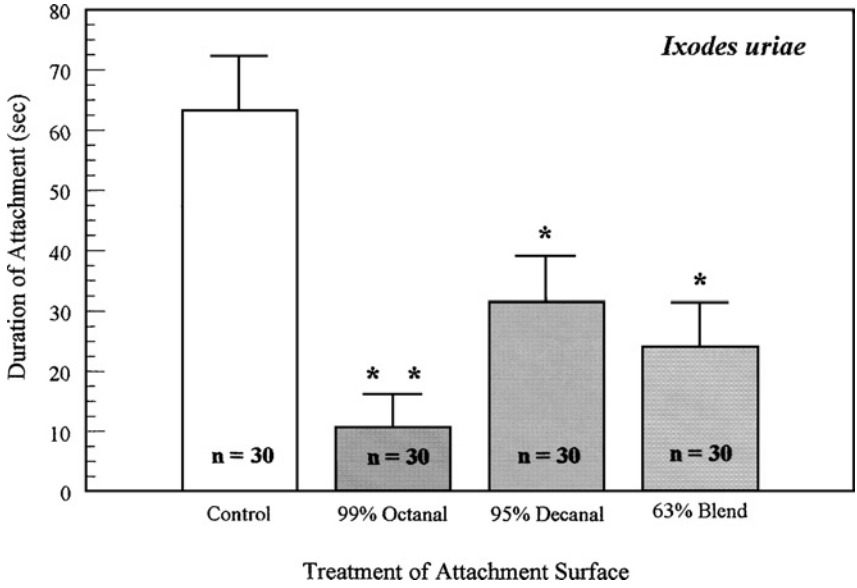

FIG. 5. Experiment III: Repellency of crested auklet odorant constituents to nymphs of *Ixodes uriae* ticks. A single asterisk indicates significance at $P < 0.05$. Two asterisks indicate significance at $P < 0.01$. The mixture of odor constituents at 63% concentration in ethanol was as repellent as nearly pure decanal.

DISCUSSION

Our combined studies suggest that the aldehyde odorants of crested and whiskered are truly species-specific and not the result of differences in location, year of collection, or diet. We are continuing to investigate the intraspecific variation in *Aethia* odorants, but at present the variation within species is less than the variation between species. As previously noted, we have also isolated the same crested auklet odorant constituents from a gland-like structure (Douglas et al., 2001).

Our chemical analysis differs slightly from Hagelin et al. (2003). They reported higher relative abundances for two oxidation products—hexanoic acid and octanoic acid. This difference is likely due to air oxidation of the aldehydes (Loudon, 2002). Hagelin et al. (2003) also reported trace compounds not discussed in our report. Those differences may have been the result of different methods for odorant collection (Raguso and Pellmyr, 1998).

Aethia aldehyde odorants appear to function as ectoparasite repellents (Douglas et al., 2001). Constituents of the crested auklet odorant repel ectoparasites, and mixtures may have synergistic properties. In plumage these

repellents may interfere with parasitism by ticks and lice. *Ixodes uriae* ticks occur in high densities at auklet colonies. *Ixodes* ticks serve as vectors for pathogens such as *Borelia* (Olsén et al., 1993), and infestations have been associated with increased mortality at some seabird colonies (Bergström et al., 1999). Chewing lice (Order Phthiraptera) are also abundant in some cases, and heavy louse infestations are known to reduce fitness in birds (Clayton, 1990). Louse abundance (Genera *Saemundssonia, Quadraceps, Austromenopon*) can exceed 200 individuals on some chicks and adults. Conversely, many adults within a population have few lice. For example, at Talan Island in 1988, lice were only found in abundance on crested auklet fledglings or chicks (11 infected, $N = 33$). No lice were observed on 80 adult crested auklet males and females by careful visual inspection (E. Hoberg, U.S. National Parasite Collection). Individuals with heavy louse infestations may serve as parasite reservoirs in avian populations, and chemical defense may serve to reduce louse transmission in highly social species like the crested auklet.

The crested auklet odorant may function as a signal of mate quality (Douglas et al., 2001). We have not noted sex-specific differences in chemistry of the auklet odorant, but we suspect that one important component of the signal is quantitative. Crested auklets that produce more of the aldehyde odorant are likely to repel ectoparasites more effectively in a dose-dependent fashion, and this would portend benefits for potential mates. Parasites harm hosts by diverting resources and acting as vectors of disease (Møller et al., 1999). Fitness benefits of parasite-free mates are lower susceptibility to disease and less likelihood of parasite transmission to mates and offspring (Loye and Carroll, 1995; Møller et al., 1999). Crested auklets may evaluate mate quality on the basis of chemical potency, and courtship, behaviors could provide an opportunity for assessment. During courtship, crested auklets bury their bills in the nape feathers of prospective mates—"ruff-sniff" behavior (Jones, 1993; Hunter and Jones, 1999; Hagelin et al., 2003). Preliminary evidence suggests that crested auklets orient to constituents of thier odorant (Douglas et al., 2002; Hagelin et al., 2003) indicating that they may be capable of smelling the aldehydes they produce.

The *Aethia* aldehyde odorants offer clues to unraveling the complex phylogeny of the true auklets. Molecular evidence suggests that *Aethia* auklets speciated rapidly as part of an adaptive radiation, but the evidence falls short of clarifying the interspecific relationships within this genus (Friesen et al., 1996). Gaston and Jones (1998) suggested that crested and whiskered auklets are more closely related based upon shared traits. Our results support and elaborate upon this working hypothesis. Characteristics of the crested and whiskered auklet odorants suggest an evolutionary divergence. Both species produce decanal in their odorant. However, feather extracts of whiskered auklets differ from those of the crested auklet in three ways: (1) dominance of odd-numbered aldehydes; (2) lack of unsaturated aldehydes; (3) presence of hexadecanol. These differences suggest

species-specific biosynthetic or sequestration pathways for the *Aethia* auklet odorants. The pattern of divergence in chemical odorants is paralleled by patterns of divergence in plumage ornaments and courtship behavior (Gaston and Jones, 1998; Hunter and Jones, 1999). Least and parakeet auklets lack the aldehyde odorant, suggesting they diverged at a different stage in the phylogeny.

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NITROGEN-INDUCED CHANGES IN PHENOLICS OF *Vaccinium myrtillus*—IMPLICATIONS FOR INTERACTION WITH A PARASITIC FUNGUS

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Abstract—The effects of nitrogen (N) fertilization on the phenolic status of *Vaccinium myrtillus* leaves were studied to assess whether N amendment affects the potentially defensive phenolic metabolites in a way that could have consequences for the interaction with a parasitic fungus (*Valdensia heterodoxa*). Healthy (symptomless) and *V. heterodoxa*-infected leaves were collected from plants grown in the understorey of a boreal coniferous forest, where they received no additional N or either a moderate or a high dose of N fertilizer. Leaf samples were taken during a single growth season and analyzed for individual phenolics using HPLC. The effect of a moderate N dose on the concentration and content of phenolics was in most cases nonsignificant. In contrast, the high N dose resulted in pronounced effects. In healthy leaves, N fertilization reduced concentration of three of five individual phenolics. Moreover, fertilization with high dose of N accompanied by infection by *V. heterodoxa* often increased the concentration and content of phenolics as compared to unfertilized plants. Addition of N had no significant effect on the growth of the analyzed *V. myrtillus* leaves, and the N-induced variation in phenolic levels seemed to be due to changed rate of their production. The concentration and content of phenolic metabolites in healthy leaves collected from unfertilized plots fluctuated compound-specifically during the growth season, and the phenolic responses to N and infection showed temporal and compound-specific variations.

Key Words—Boreal forest, bilberry, chemical defense, disease resistance, nitrogen fertilization, *Valdensia heterodoxa*.

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INTRODUCTION

Nitrogen (N) is one of the limiting factors for primary production of plants, and its availability also affects the growth and reproduction of herbivores and microorganisms. Anthropogenic activities (e.g., industry and agriculture) often lead to increased N deposition in the environment. This is likely to have a variety of effects, both positive and negative, on the different trophic levels of terrestrial ecosystems (Aber et al., 1989; Vitousek and Howard, 1991; Lee, 1998). Ecological consequences of N deposition may be particularly profound for ecosystems that are inherently N poor and have historically low background levels of N deposition, such as dry heathlands and boreal forests (Hunter and Price, 1991; Nordin et al., 1998; Strengbom et al., 2002). In these ecosystems, addition of N leads to changes in field layer vegetation, usually involving increased abundance of fast-growing graminoids (grasses) at the expense of slow-growing and nutrient conserving species, such as ericaceous dwarf shrubs (e.g., *Calluna* and *Vaccinium* species) (Aerts and Berendse, 1988; Aerts et al., 1990; Strengbom et al., 2002, 2003). This transition may be mediated, at least in part, by N increasing the susceptibility of the slow-growing species to damage by abiotic factors (e.g., frosts or drought), herbivores, or parasites (Heil and Diemont, 1983; Strengbom et al., 2002). This, in turn, reduces competitive ability and causes perturbation of the coverage, which allows fast-growing species to establish and take over (Aerts et al., 1990; Lee, 1998, and references therein; Strengbom et al., 2002, 2003).

In the understorey of a Norway spruce-dominated boreal forest, N addition decreases the abundance of *Vaccinium myrtillus* L. (bilberry), while that of a grass species, *Deschampsia flexuosa* L., increases (Strengbom, 2002; Strengbom et al., 2002). Concurrently, an increase in the incidence of a fungal parasite, *Valdensia heterodoxa* Peyr., on *V. myrtillus* leaves has been recorded on N-fertilized plots (Strengbom et al., 2002). The fungus causes brown spot disease, which may lead to premature shedding of infected leaves and patchiness of *V. myrtillus* cover. The fungus overwinters on fallen leaves as sclerotia, from which the fruit bodies develop in the following spring. The initial infection of host plants takes place in the early summer, and the infection develops from conidia, which are formed on necrotic lesions (Norwell and Redhead, 1994; Vogelgsang and Shamoun, 2002). The phytochemical basis of the observed N-induced increase in the susceptibility of *V. myrtillus* to *V. heterodoxa* is not yet clear. Increased N (amino acid) concentrations of leaves in fertilized plants may at least partially explain this increase. Earlier studies have shown that concentration of glutamine increases in fertilized *V. myrtillus* plants, and spraying of leaves with glutamine solution increases the incidence of *V. heterodoxa* (Nordin et al., 1998; Strengbom, 2002; Strengbom et al., 2002). However, increased N availability may affect the phytochemical quality of plants in several ways, and we still know little about the possible N-induced

changes in other phytochemicals, which may predispose *V. myrtillus* to infestation by parasitic fungi.

The vegetative tissues of *V. myrtillus* plants contain a variety of carbon-based secondary metabolites, such as phenolic acids and flavonoids (Gallet, 1994; Fraisse et al., 1996; Witzell et al., 2003). Some of these compounds possess antimicrobial activity (Kokubun and Harborne, 1994; Ho et al., 2001; Puupponen-Pimiä et al., 2001) and are, therefore, potentially important in *V. myrtillus–V. heterodoxa* association (see also Nicholson and Hammerschmidt, 1992; Bennett and Wallsgrove, 1994; Dixon and Paiva, 1995, for general aspects of phenolics in plant defense). Increased N availability in the environment may decrease the levels of phenolic metabolites in plants (Haukioja et al., 1998; Koricheva et al., 1998). Although simple and mechanistic explanations for environmentally induced changes in allocation of resources to carbon-based defenses have been searched for during the last decades [e.g., studies testing the validity of the carbon–nutrient balance (CNB) hypothesis; Bryant et al., 1983], recent evaluations have emphasized that the production of phenolic metabolites responds to changes in nutrient availability in a highly complex manner (Haukioja et al., 1998; Koricheva et al., 1998; Penuelas and Estiarte, 1998; Jones and Hartley, 1999; Hamilton et al., 2001; Koricheva, 2002; Lerdau and Coley, 2002). For instance, the ontogenetic and phenological variation in plant phenolic metabolism (Kause et al., 1999; Riipi et al., 2002; Kleiner et al., 2003) needs to be better considered in studies addressing phenolic responses to abiotic and biotic factors. Our understanding of carbon allocation patterns under different nutrient regimes may be further refined by methodological improvements. Traditionally, predictions about treatment-induced shifts in resource allocation to secondary metabolites have been based on concentration data. Variation in concentrations of secondary metabolites across treatments may, however, be due simply to differences in plant growth and accumulation of biomass (Koricheva, 1999). Thus, it has been suggested that the measurement of simultaneous shifts in biomass production and absolute content of secondary metabolites may provide more accurate information about the mechanisms underlying changes in their concentrations (Koricheva, 1999; Koricheva and Shevtsova, 2002).

The aim of our study was to investigate the effects of N fertilization on the phenolic chemistry of healthy and *V. heterodoxa*-infected *V. myrtillus* leaves. We hypothesized that N amendment decreases the constitutive levels of defensive phenolics in leaves, or depresses the parasite-induced changes in phenolics, which could explain their increased susceptibility to fungal parasites associated with increases in N supply (cf. Nordin et al., 1998; Strengbom, 2002; Strengbom et al., 2002, 2003). To assess plant quality for parasites (and, potentially other natural enemies) at different levels of N fertilization, we analyzed changes in concentrations of phenolics. In addition, to distinguish changes in phenolic levels due to treatment-induced effects on biomass accumulation from changes in allocation patterns, we also determined the total amount of compounds per individual leaf.

The measurements were done on four occasions to study the temporal variations in phenolics in relation to the progress of the infection. This study is a part of a research project attempting to define the N effects on various phytochemical characters that are critical for the parasite resistance of *V. myrtillus*.

METHODS AND MATERIALS

Study Area and Treatments. The experimental area, Svartberget Research Station, is situated in northern Sweden, 60 km NW from Umeå (64°14′N, 19°46′E). The experimental forest is a late successional spruce (*Picea abies* L. Karst) forest, with a field layer dominated by the ericaceous dwarf shrub *V. myrtillus*. The experiment was set up as a randomized complete block design in 1996. Within each of five blocks (150 × 150 m), three 31.6 × 31.6 m plots were randomly assigned to N fertilization treatments of 0 (N0-plots, unfertilized control), 12.5 (N1-plots, moderate N level), and 50 (N2-plots, high N level) kg ha^{-1} y^{-1}. The fertilizer was given as NH_4NO_3 in the form of granules once a year (at the beginning of the growth season) for 5 years (1996–2000). In the study area, the background level of N deposition is low (about 3.5 kg N ha^{-1} y^{-1}; Strengbom, 2002). In general, the level of air-borne N deposition in Sweden has been reported to vary from 2–3 to 20 kg ha^{-1} y^{-1} in northern and southern parts, respectively (Strengbom et al., 2003, and references therein), whereas in Central Europe higher amounts have been reported (e.g., in the Netherlands from 50 to 85 kg ha^{-1} y^{-1}; Berendse et al., 1993, cited by Vitousek, 1994; Dise et al., 1998; Tietema et al., 1998). Thus, the N doses used in our study were ecologically realistic for Northern and Central European conditions.

Sample Collection. Samples were collected on four occasions during the growth season of 2000: in mid-June, mid-July, mid-August, and late August. These sampling occasions were considered to represent, respectively, the initial phase of *V. heterodoxa* infection by ascospores (mid-June), the expansive phase by the conidial infection (mid-July and mid-August), and the late phase, when the first overwintering structures are initiated (late-August). Within each plot, several ramets of *V. myrtillus* were chosen randomly. From these ramets, we haphazardly selected one ramet without visible symptoms of infection by *V. heterodoxa* (June–July) or with less than 10% of infection (August) and one ramet with visible symptoms of infection (>50% of infected leaves). The distance between the two ramets did not exceed 2 m to reduce possible chemical variation due to patchiness of soil and microclimate. A leaf without visible symptoms (healthy) was chosen from a healthy ramet, and a leaf bearing necrotic lesions symptomatic of *V. heterodoxa* infection (infected) was collected from the other ramets for analysis. Care was taken to choose leaves of similar developmental stage (i.e., leaves in a phase where they were no longer actively expanding, but not yet senescent). The presence of parasites or damage from herbivores on other leaves

during the study season or preceding growth seasons was regarded as background variation and not recorded in this study. The analysis of single leaves rather than whole shoots was preferred because of the difficulty of finding whole shoots free of parasite infestations during the entire experimental period. During the first sampling occasion, parasite frequency on the plots was still low, and in both June and July whole annual shoots in which none of the leaves had visible symptoms could be found. However, during August this was often impossible, because of the high incidence of parasites. The disease incidences for the N treatments were measured during 1996–2000 (Strengbom, 2002).

Collected leaves were air-dried in a well-ventilated room at ambient temperature. After this, the dry leaves were kept in a desiccator for 48 hr, and each was weighed. A higher drying temperature (e.g., 105°C) for dry weight determination was not used because of the risk of causing changes in phenolics (Waterman and Mole, 1994). After weighing, the necrotic tissue was separated with a scalpel from the symptom-bearing leaves and discarded to ensure that the analyzed tissue represented living cells. Leaves were stored at −20°C and equilibrated to room temperature in a desiccator for 24 hr before analysis.

Extraction and Analysis of Phenolics. Phenolics were analyzed using an HPLC system equipped with a photodiode array detector according to the method described by Witzell et al. (2003). The methanol extracts contained several phenolics, of which five compounds, which represented different types of phenolics, were selected for a more detailed analysis. On the basis of retention times and specific UV-spectra (200-400 nm), which were compared to authentic standards, these compounds were identified as chlorogenic acid, catechin, arbutin, *p*-coumaric acid, and quercetin-3-glucoside.

Data Analyses. We estimated the effects of N fertilization, *V. heterodoxa* infection, and sampling time on phenolic compounds by using mixed analysis of variance with SAS statistical software (proc MIXED, SAS Release 8.2). Leaf dry weight and the concentrations and contents of the selected phenolic compounds were the dependent variables. The N fertilization treatment (with three levels) served as a fixed factor and was further decomposed into orthogonal contrasts testing for linear and quadratic trends. Since the area covered by the individual clones of *V. myrtillus* can be extensive (up to 5–15 m diam; Ritchie, 1956; Flower-Ellis, 1971), the diseased and healthy leaves may have originated from the same clone of *V. myrtillus* and could not be considered as independent samples. Therefore, infection was treated as a split-plot factor and the fertilization as a whole-plot factor. The random factors included block and block × fertilization. Samples that were successively harvested from the same microhabitats of the same plots were also considered as dependent samples, and the sampling time was treated as a repeated-measures factor. The covariance matrix of the model was chosen by fitting several different correlation models and comparing their respective Akaike's Information criteria. A mixed ANOVA model was used with the

ddfm=satterth option, invoking the Satterthwait approximation (Schabenberger and Pierce, 2002). Mean comparisons between different levels of treatments and different sampling dates were performed by Tukey–Kramer and Dunnett's tests (LSMEANS/diff option in proc MIXED; SAS Institute, 1999). In addition, in the case of significant interactions between main factors, the simple effects were studied by using LSMEANS/slice option (proc MIXED; SAS Institute, 1999). Prior to analysis, each data set was tested for normality (Shapiro–Wilk's test) and homogeneity of variances (Levene's test) to verify that it conformed to the assumptions of ANOVA, and the data were transformed when necessary. Untransformed values are presented in the figures.

Changes in leaf growth over the season, and in the concentration and content of phenolics, were visualized and interpreted with the help of graphical vector analysis (GV analysis; Koricheva, 1999; Koricheva and Shevtsova, 2002; see also Figure 2f for interpretation of GV diagrams). For this, mean concentrations and contents of individual phenolics for each treatment and sampling occasion were expressed as values relative to the corresponding values of the control (healthy leaves from unfertilized plots). The relative changes in concentrations and contents of the phenolic were plotted against each other on a vector diagram. The mean leaf weights represented the inverse of the slope factor. The effects of the treatments were expressed by vectors, connecting values for the control ($x = y = z = 1$) with the values for the treatments. Seasonal variations for each individual phenolic compound studied were analyzed in the healthy leaves from unfertilized plots relative to the levels observed at the first sampling occasion (mid-June) (for more details on vector diagrams construction and interpretation, see Koricheva, 1999).

RESULTS

Leaf Dry Weight. There were no significant effects of N, sampling time or infection on the dry weight of leaves, although the interactive effect of infection and sampling time on leaf weight was significant (Table 1). In the healthy leaves from the unfertilized plots, we found no changes in the dry weight of the samples collected between mid-June and late August [$F(3, 36) = 0.63$, $P = 0.600$]. Infected leaves were marginally lighter than healthy leaves in June [$F(1, 80.8) = 3.52$, $P = 0.064$], but in mid-August infected leaves were heavier than the healthy ones [$F(1, 81.5) = 6.05$, $P = 0.016$] (Figure 1).

Seasonal Changes in Phenolics in Healthy Leaves from Unfertilized Plots. A significant sampling time effect was found on individual phenolic concentration and on the absolute content of all phenolics, with the exception of quercetin-3-glucoside (Table 1). The GV analysis also indicated that there were seasonal variations in individual phenolic compounds (Figure 2). For instance, the level of arbutin was lowest in July, but increased close to the initial (June) level during August (Figure 2c). Both the concentration and content of the other four phenolics

TABLE 1. RESULTS OF REPEATED-MEASURES ANOVA FOR THE EFFECTS OF NITROGEN FERTILIZATION (N), *Valdensia heterodoxa* INFECTION (INF) AND SAMPLING TIME (TIME) ON LEAF DRY WEIGHT (DW), AND CONCENTRATIONS AND LEAF CONTENTS OF INDIVIDUAL PHENOLIC COMPOUNDS OF *Vaccinium myrtillus*

			Changes in concentration (P)				
Effect	*df*	Changes in leaf d.w.	Chlorogenic acid	Catechin	Arbutin	Quercetin-3-glycoside	*p*-Coumaric acid
N	2	0.449	0.026	0.981	0.046	0.180	0.039
N quadratic	1		0.974	0.862	0.861	0.728	0.220
N linear	1		0.007	0.930	0.014	0.073	0.026
Inf	1	0.686	0.412	0.232	0.558	<0.001	0.027
N*Inf	2	0.422	0.327	0.740	0.399	0.150	0.055
N*Inf quadratic	1		0.651	0.520	0.995	0.656	0.770
N*Inf linear	1		0.157	0.681	0.178	0.058	0.017
Time	3	0.220	0.035	<0.001	0.002	0.033	0.017
N*Time	6	0.770	0.100	0.149	0.533	0.409	0.922
Inf*Time	3	0.023	0.699	0.486	0.222	0.744	0.958
N*Inf*Time	6	0.811	0.745	0.067	0.245	0.357	0.553
Transf.[a]		log	arsin(sqrt)	log	log	log	arsin(sqrt)
Covar.[b]		ar(1)	Sp(exp)	ar(1)	ar(1)	ar(1)	vc
			Changes in content (P)				
N	2		0.391	0.664	0.561	0.672	0.479
N quadratic	1		0.706	0.574	0.980	0.973	0.266
N linear	1		0.190	0.485	0.286	0.383	0.668
Inf	1		0.351	0.475	0.546	<0.001	0.031
N*Inf	2		0.191	0.825	0.178	0.052	0.014
N*Inf quadratic	1		0.825	0.724	0.666	0.359	0.259
N*Inf linear	1		0.071	0.618	0.071	0.023	0.007
Time	3		0.028	<0.001	0.003	0.084	0.010
N*Time	6		0.101	0.240	0.591	0.513	0.981
Inf*Time	3		0.133	0.407	0.194	0.288	0.196
N*Inf*Time	6		0.884	0.065	0.541	0.375	0.746
Transf.			arsin(sqrt)	log	arsin(sqrt)	log	arsin(sqrt)
Covar.			vc	ar(1)	ar(1)	ar(1)	vc

[a]Transformation of the data; [b]The covariance matrix of the model (ar(1) – first-order autoregressive; Sp(exp) – exponential; vc – variance components).

were highest in mid-July (catechin and quercetin-3-glucoside; Figure 2b and d) or mid-August (chlorogenic acid and *p*-coumaric acid; Figure 2a and e). After these dates, levels were reduced, and by late August they had returned close to their initial (June) levels (Figure 2). The magnitude of seasonal changes was highest for catechin, with a twofold increase in concentration from mid-June to mid-July.

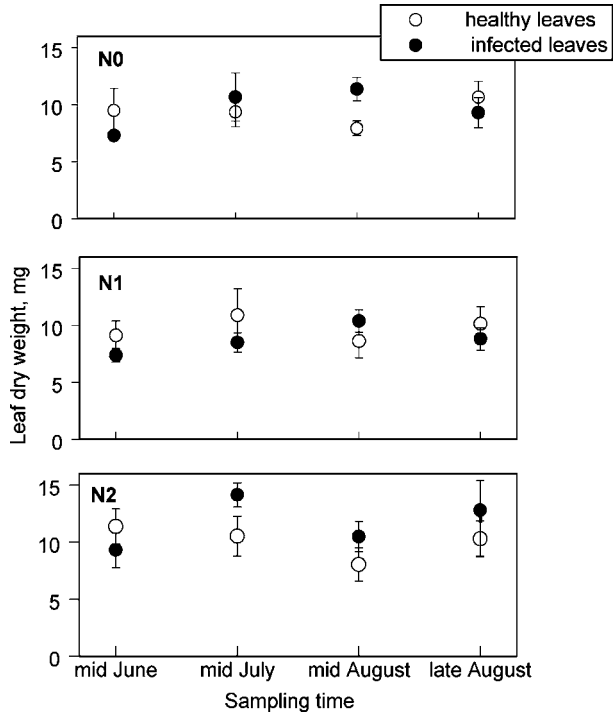

FIG. 1. Dry weight (mg) of healthy and infected leaves of *Vaccinium myrtillus* collected from plots with different levels of N fertilization (N0 – no additional N; N1 – addition of 12.5 kg N ha^{-1} y^{-1}; N2 – addition of 50 kg N ha^{-1} y^{-1}) collected on four occasions during the growth season. Values are means ± SE.

Effects of N Fertilization on Phenolics of Healthy Leaves During the Growth Season. The concentrations of arbutin, chlorogenic acid, and *p*-coumaric acid were reduced by N fertilization (Table 1, Figures 3–7). In healthy plants, N amendment reduced the concentration of arbutin in mid-June (Figure 3) and chlorogenic acid (Figure 4) and *p*-coumaric acid (Figure 5) in mid-July and mid-August (Table 1). Differences in phenolic concentration were detected most often between control and N2-treated plants (Figures 3–7). However, the concentrations of chlorogenic acid (Figure 4) and catechin (Figure 6) in healthy leaves were reduced by the moderate level of N fertilization in mid-July.

The absolute contents of any of the individual phenolics were generally not significantly affected by N additions (Table 1), although the changes generally followed the same trend as that of their concentration (Figures 3–5). However, N significantly reduced the content of chlorogenic acid on N2-treated plots in mid-July (Figure 4).

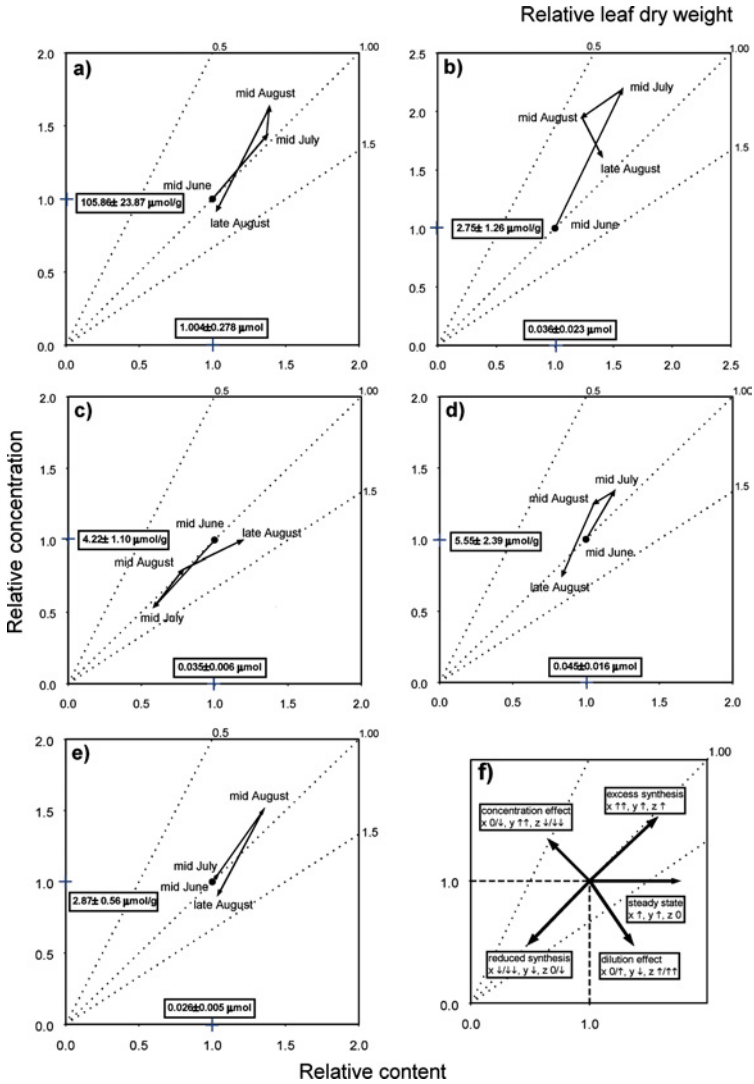

FIG. 2. Seasonal changes in concentrations and contents of individual phenolic compounds in healthy leaves of *Vaccinium myrtillus* on control (unfertilized) plots relative to the level of these compounds in mid-June: (a) chlorogenic acid; (b) catechin; (c) arbutin; (d) quercetin-3-glucoside; (e) *p*-coumaric acid; and (f) an interpretation key to GV analysis. For each individual phenolic compound, concentration and content are expressed as values relative to the levels observed at the first (reference) sampling occasion (mid-June). See Methods and Materials for more details on vector diagram construction. The values for the absolute concentrations (μmol/g DW $\pm$ SE) and leaf contents (μmol/leaf $\pm$ SE) of the phenolic compounds are indicated along the corresponding axes for the reference sampling (mid-June).

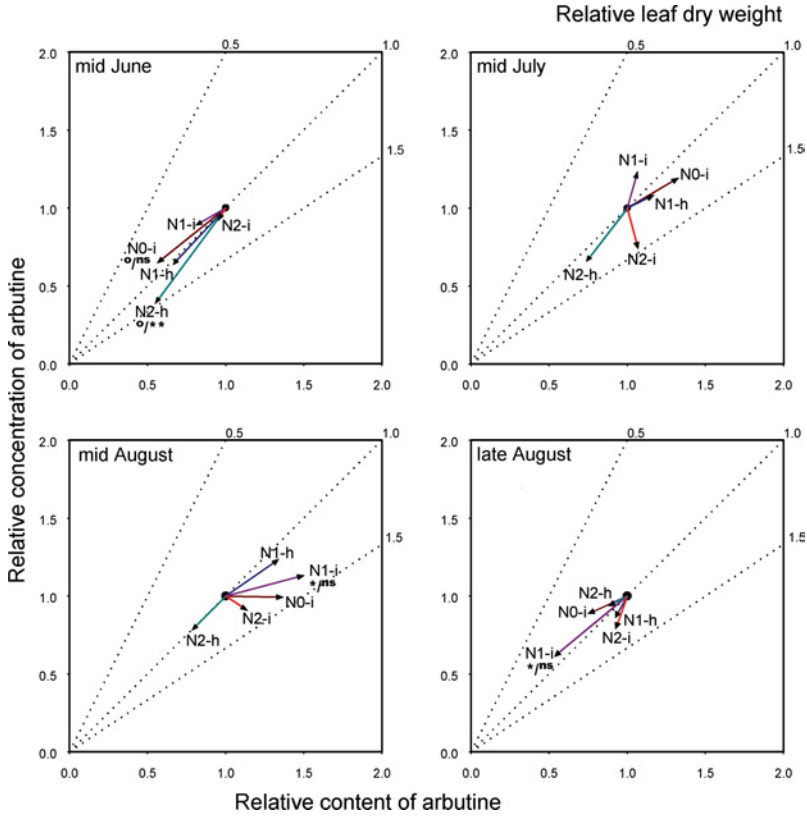

FIG. 3. Vector diagrams of effects of N fertilization (N0 – control; N1 – addition of 12.5 kg N ha^{-1} y^{-1}; N2 – addition of 50 kg N ha^{-1} y^{-1}) on the concentration and content of arbutin in *Vaccinium myrtillus* measured in healthy leaves (−h) and leaves infected by *Valdensia heterodoxa* (−i) on four occasions during the growth season. For each sampling occasion, effects of treatments are represented by vectors, connecting values of the control (N0-h), for which ($x = y = 1$), with the values of the treatments expressed as values relative to the corresponding value of the control. See text and Figure 2f for more details on vector diagram construction. The significance of differences between treatments and the control is indicated by asterisks (for content/concentration, as marked), where ns, o, ∗, and ∗∗ indicate $P > 0.1$, $0.05 < P < 0.1$, $P < 0.05$, and $P < 0.01$, respectively (Dunnett's test).

Effects of N Fertilization on Infection-Associated Changes During the Growth Season. Infection by *V. heterodoxa* significantly affected both the concentration and absolute content of quercetin-3-glucoside and *p*-coumaric acid (Table 1). However, the magnitude and direction of phenolic changes in *V. heterodoxa*-infected leaves depended on the fertilization level (Figures 5 and 7), as indicated

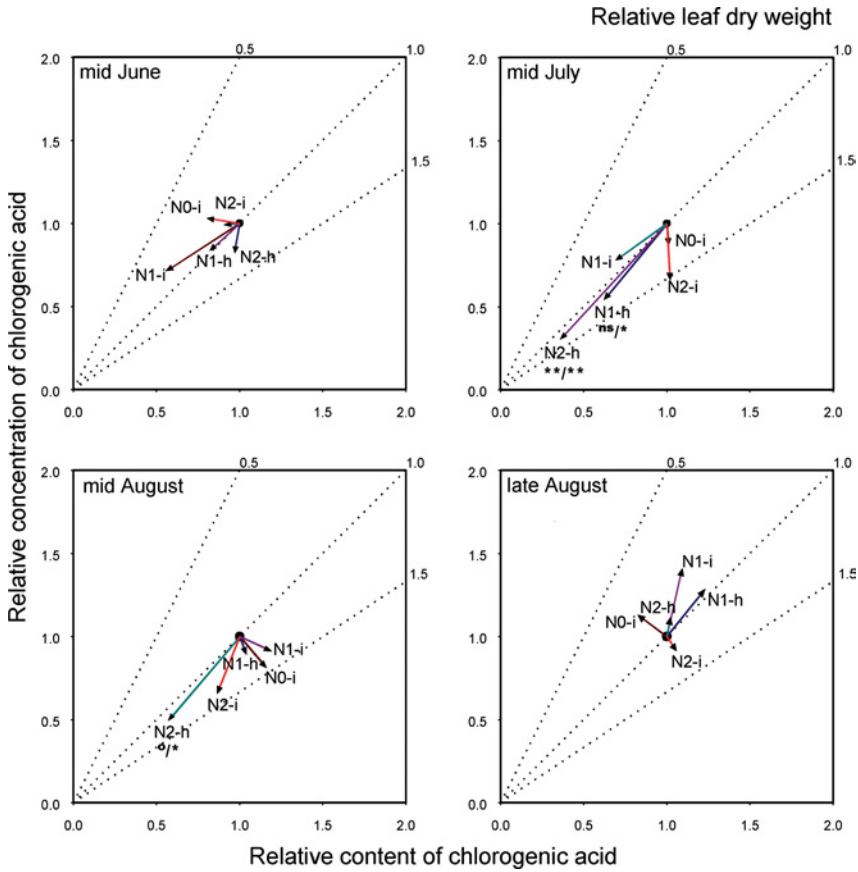

FIG. 4. Vector diagram of effects of N fertilization on the concentration and content of chlorogenic acid in *Vaccinium myrtillus* measured in healthy leaves and leaves infected by *Valdensia heterodoxa* on four occasions during the growth season. See Figure 3 for key to the treatment codes.

by the significant or marginally significant interactions between the linear trend for N and infection (Table 1).

In general, the concentration and absolute content of most phenolics tended to increase in infected leaves of plants growing on N2-plots, but the timing of increases seemed to vary compound-specifically. For instance, the concentration and content of *p*-coumaric acid and quercetin-3-glucoside were elevated in infected leaves collected from N2-plots in mid-July and August (Figures 5 and 7), and those of arbutin and chlorogenic acid in mid-June and July (Figures 3 and 4), respectively (Tukey–Kramer test, $P < 0.05$). The changes in concentration and

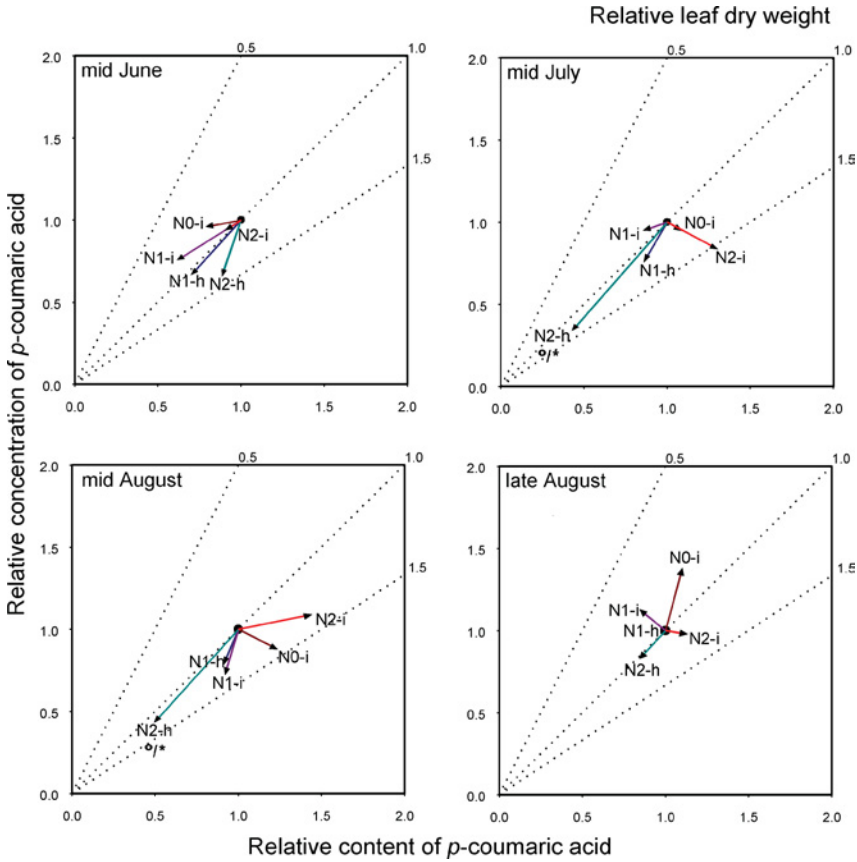

FIG. 5. Vector diagram of effects of N fertilization on the concentration and content of *p*-coumaric acid in *Vaccinium myrtillus* measured in healthy leaves and leaves infected by *Valdensia heterodoxa* on four occasions during the growth season. See Figure 3 for key to the treatment codes.

content of catechin in infected leaves in mid-August, accompanied by increase in leaf biomass (Figure 6) suggest that excess catechin synthesis occurred in infected leaves during this period. This trend was significant not only in N2-treated but also in unfertilized plants. These results indicate that in most cases, significant changes in the allocation of resources to phenolic acids (chlorogenic acid and *p*-coumaric acid) and polyphenols (quercetin-3-glucoside and catechin) in response to N treatments and infection occurred during July and mid-August (Figures 3–7). For arbutin, on the other hand, N-induced reduction in allocation had already occurred in June (see healthy leaves of N2-treated plants in Figure 3).

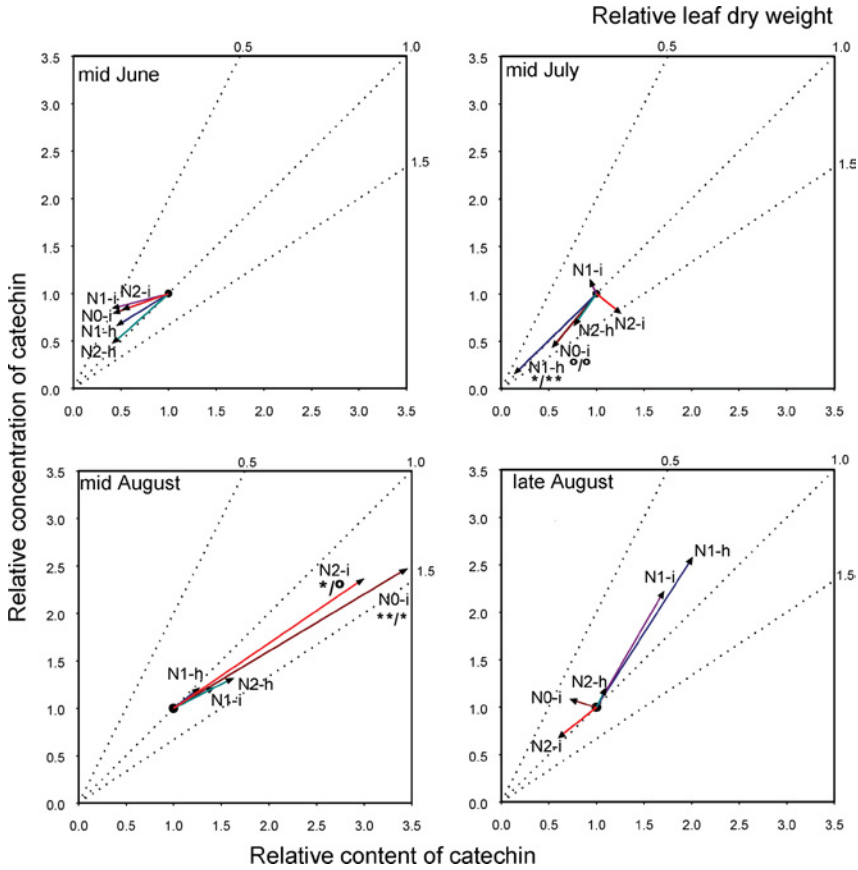

FIG. 6. Vector diagram of effects of N fertilization on the concentration and content of catechin in *Vaccinium myrtillus* measured in healthy leaves and leaves infected by *Valdensia heterodoxa* on four occasions during the growth season. See Figure 3 for key to the treatment codes.

DISCUSSION

Fertilization with N usually accelerates the vegetative growth of plants, so the developmental stage and size of plants under different N regimes may vary considerably (Gebauer et al., 1998). Phenolic levels also show developmental and temporal variations (Kause et al., 1999; Riipi et al., 2002; Kleiner et al., 2003). Thus, careful selection of plant material is necessary to avoid comparisons of plants or plant tissues at different developmental stages (Coleman et al., 1994). The fact that we found no significant effect of N treatment, sampling time, or

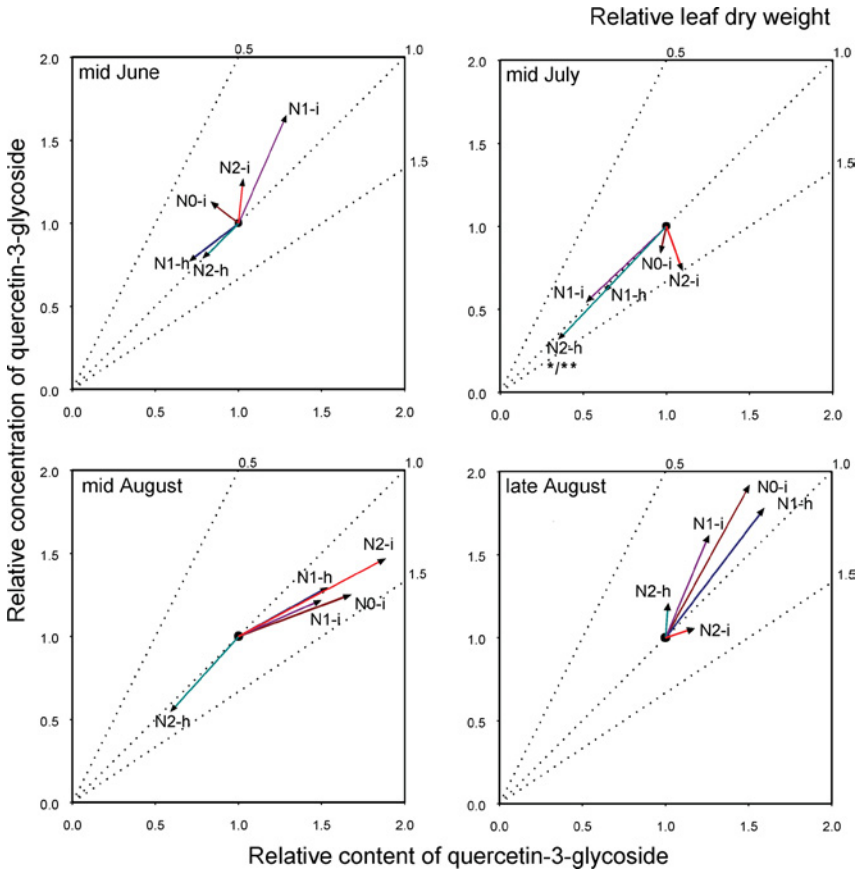

FIG. 7. Vector diagram of effects of N fertilization on the concentration and content of quercetin-3-glucoside in *Vaccinium myrtillus* measured in healthy leaves and leaves infected by *Valdensia heterodoxa* on four occasions during the growth season. See Figure 3 for key to the treatment codes.

infection on leaf dry weight suggests that the leaves collected for analyses at different times had reached a similar growth phase and size, and, thus, the chemical data obtained across the sampling period were comparable. Lack of N effect on leaf size is in agreement with results of other studies that have shown nonsignificant increase in growth (shoot biomass and length) of *V. myrtillus* in response to N amendment (Richardson et al., 2002; Strengbom, 2002; Strengbom et al., 2002). The smaller size of infected leaves in June suggests that infection had a negative initial impact on leaf growth. This may reflect an increased investment of resources to processes other than growth, for instance to respiration, which is often

accelerated during parasite infection (Simons et al., 1999). The higher dry weight of infected leaves later in the growth season may reflect increased accumulation of structural components and strengthening of the cell walls as a protective response against parasite invasion.

Our results indicate that marked within-seasonal variation occurred in the levels of individual phenolic compounds in healthy leaves of unfertilized *V. myrtillus*. Dry weights in mid-June and late August were similar, suggesting that the shifts in phenolic levels were not due to the distribution of a constant amount of phenolics in a varying amount of biomass but rather affected by other processes. The size of the pool of any phenolic compound may be affected by production, catabolism, turnover, and transport (Reichardt et al., 1991). However, recent studies indicate that some plant phenolics may be more stable than previously thought, and that they often accumulate in the cells in which they are synthesized (Ruuhola et al., 2001, and references within). Thus, we suggest that the observed within-seasonal increase in concentration and content of phenolics may be mainly due to within-seasonal increase in their rate of production (cf. Koricheva, 1999; Koricheva and Shevtsova, 2002), indicating that in *V. myrtillus* phenolic biosynthesis is actively regulated in response to inherent or external cues. The fluctuation in chemical quality of leaf tissues may act as a constitutive buffer against damage caused by herbivore and pathogen attacks, which peak at different times during the season (cf. Osier et al., 2000).

We found that N fertilization significantly reduced the concentrations of chlorogenic acid, arbutin, and *p*-coumaric acid in *V. myrtillus* leaves, especially in healthy leaves. Considering the nonsignificant N effect on leaf dry weight, these results may suggest that N-induced reduction in concentrations of these compounds were primarily caused by reductions in their production (cf. Koricheva, 1999; Koricheva and Shevtsova, 2002). This is in agreement with earlier results concerning N effect on phenolics in *V. myrtillus* leaves (Grellmann, 2001) and with the general tendency of reduction in phenolic production in woody plants in response to fertilization (reviewed by Haukioja et al., 1998; Koricheva et al., 1998). However, levels of two of the other analyzed compounds, quercetin-3-glucoside and catechin, were not significantly affected by N, indicating that individual phenolics in *V. myrtillus* leaves show variations in response to N amendment. Observed variation in response of individual phenolics to N fertilization may be explained by differences in their chemical structures and biosynthetic pathways (Haukioja et al., 1998; Koricheva et al., 1998). The lack of an unambiguous N effect on quercetin-3-glucoside and catechin may also indicate that their levels in *V. myrtillus* were more strongly influenced by factors other than N availability. For example, flavonol glycosides are important in plant protection against UV light (Booij-James et al., 2000), and light conditions may be a regulator of their synthesis and accumulation.

Our results suggest that phenolic metabolism of *V. myrtillus* was not markedly altered by the moderate N dose applied in our 5-year study. Possibly, vegetative

reproduction provides *V. myrtillus* with buffering mechanisms that attenuate the effects of moderate N addition on secondary metabolism. Sharing of photosynthates and nutrients among ramets is crucial for the success of clonal plants (Orians and Jones, 2001). Nevertheless, with the higher N dose, more pronounced alterations in the secondary metabolism were detected, suggesting that any potential buffering capacity is exceeded at a level of 50 kg ha^{-1} y^{-1}, which is high for boreal forest ecosystems, but realistic for Central Europe. This finding accentuates the importance of cautiously interpreting results of studies (e.g., those testing the validity of the predictions of the CNB hypothesis), where the levels of nutrition manipulations are outside the regime that the plants have evolved to "expect" in their natural environments.

In vitro effects of these compounds on growth and development of *V. heterodoxa* are the subjects of future studies. However, chlorogenic acid, catechins, quercetin, and arbutin (or hydroquinone released from it) have been pointed out as antimicrobial agents in earlier literature (Kokubun and Harborne, 1994; Ho et al., 2001; Puupponen-Pimiä et al., 2001). We, thus, anticipated that our compounds were potential antifungal agents in the *V. myrtillus–V. heterodoxa* association. We expected that their constitutive or induced levels might be lowest in fertilized plants, which show the lowest resistance to *V. heterodoxa* parasite (Strengbom, 2002; Strengbom et al., 2002). Indeed, we found an N-induced reduction in concentrations of individual phenolics in healthy leaves. If the analyzed phenolics have activity as constitutive antifungal agents in *V. myrtillus* leaves, such reductions would partly explain the increased disease susceptibility reported in earlier studies in plants growing on the same N-fertilized plots (Strengbom et al., 2002).

Interestingly, we observed higher concentrations and contents of some individual phenolics in infected leaves, mainly at the N2 level. Parallel results have been reported by Strengbom et al. (2003), who found that the N2 treatment tends to increase N concentration in *V. myrtillus* plants whereas C concentrations remain the same as in unfertilized controls, or are elevated by N addition (Strengbom et al., 2003; J. Strengbom, unpublished results). A plausible biochemical mechanism behind the phenolic increases at high N treatment might be that the higher N dose resulted in enhanced C fixation or stimulated phenolic synthesis, possibly because of increased levels of photosynthetic enzymes and amino acid precursors for phenolic synthesis (Gebauer et al., 1998; Haukioja et al., 1998; Jones and Hartley, 1999).

The compound-specific and within-seasonal variation in phenolic levels imply that an individual phenolic compound may be functional (i.e., have either negative or positive effects on the parasite) only at a certain phase of an infection. For instance, the phenolic glycoside arbutin responded most markedly during the initial, ascospore infection, while the phenolic acids and the flavonoid quercetin-3-glucoside appeared to be involved in defensive or stress responses induced by

the conidial stage of the fungus. The increased synthesis of catechin in infected leaves in mid-August, on the other hand, implies that this compound (and the proanthocyanidins derived from it) may interact with the initiation of fungal overwintering structures (sclerotia). Future studies with controlled inoculations and *in vitro* tests assessing the direct effects of phenolics on the growth and development of *V. heterodoxa* should provide the necessary confirmation or challenge to these hypotheses.

In our study, the lack of controlled inoculations precludes detection of any causal relationships between fungal infection and phenolic induction. Thus, the possible connections between the growth and development of *V. heterodoxa* and the observed N-dependent increases in phenolics in infected *V. myrtillus* leaves are not yet clear. Phenolic increases in infected plants may represent a defensive response (Nicholson and Hammerschmidt, 1992; Dixon and Paiva, 1995). However, since the highest incidence of *V. heterodoxa* has been reported for the N2 treatment (Strengbom, 2002; Strengbom et al., 2002), contribution of the above-mentioned phenolic increases under high N amendment to protection against *V. heterodoxa* appears limited. Rather, it seems likely that the observed phenolic increases were a general stress response to infection that was conditional on the high N amendment in the environment. Since the fungal infections were not experimentally manipulated, and since the detected phenolic responses in infected leaves may represent local, short-term responses to the parasitic fungus, assessment of their broader ecological importance for the herbaceous layer of the boreal forest ecosystem is not straightforward. Nevertheless, because all shifts in plant chemistry may change the quality of plants for consumers (cf. Ball et al., 2000; Hambäck et al., 2002), it is conceivable that quantitative changes in phenolics of infected *V. myrtillus* leaves could have consequences for other pathogens or herbivores as well. Moreover, the changed phenolic content of prematurely fallen, diseased leaves may affect various soil-mediated ecological relationships and the microbial flora in the soil (Gallet, 1994; Nilsson et al., 1998; Schimel et al., 1998).

In summary, our results indicate that N-induced reductions in the constitutive levels of phenolics may be linked to increased susceptibility of boreal *V. myrtillus* to *V. heterodoxa* infection under N amendment (cf. Strengbom et al., 2002). They also confirm that the magnitude and direction of abiotic and biotic effects on phenolic metabolism of *V. myrtillus* leaves have a marked seasonal component. Moreover, the changes in levels of certain phenolics in *V. myrtillus* leaves occur mainly in plants treated with an N loading that is over 10-fold compared to the natural N level that the plants are adapted to "expect." These results emphasize the importance of sampling on multiple occasions, as well as recognizing the natural N regimes of the studied plant species, in any study that addresses the ecological consequences of environmentally induced alterations in plant phenolic metabolism.

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ANTITERMITIC AND ANTIFUNGAL ACTIVITIES OF ESSENTIAL OIL OF *Calocedrus formosana* LEAF AND ITS COMPOSITION

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Abstract—*Calocedrus formosana* Florin (Cupressaceae) is an endemic tree species in Taiwan; its timber is recognized for natural decay resistance. To examine the antitermitic and antifungal activities of leaf essential oil and its main constituents, *C. formosana* leaves were extracted and the essential oils analyzed by GC-MS. Bioactivity tests against the termite *Coptotermes formosanus* demonstrate that the LC_{50} value of leaf essential oil is 27.6 mg/g. Furthermore, exposure to T-muurolol caused 100% mortality at a dosage of 5 mg/g after 14 d. Leaf oil constituents displayed activity against four fungi, *Lenzites betulina*, *Pycnoporus coccineus*, *Trametes versicolor*, and *Laetiporus sulphureus*. Two compounds, α-cadinol and T-muurolol, exhibited the strongest antifungal activity. The LC_{50} values of α-cadinol against *L. sulphureus*, *L. betulina*, and *T. versicolor* are 9.9, 28.6, and 30.4 μg/ml, respectively.

Key Words—*Calocedrus formosana*, leaf, essential oil, GC-MS, *Coptotermes formosanus*, antitermitic activity, antifungal activity, α-cadinol, T-muurolol.

INTRODUCTION

Wood, a naturally occurring polymer composite, is mainly composed of cellulose, hemicelluloses, lignin, and extractives. Due to its biological nature, unprotected wood is susceptible to discoloration and biological deterioration, which reduce its mechanical and physical properties (Chang et al., 2002). Developing methods that prolong the service life of wood has always been the interest of wood researchers. From an environmental perspective, finding naturally occurring constituents in

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highly durable tree species and understanding their mechanisms are the most appropriate approaches to achieving wood protection while preserving the environment (Chang et al., 2000). In recent years, many studies have investigated the relationship between wood properties and wood essential oils (Kinjo and Yata, 1986; Kondo and Imamura, 1986; Asada et al., 1989; Yoneyama et al., 1990; Nabeta et al., 1992; Morita et al., 1997; Chang et al., 2001a,b; Chang and Cheng, 2002). Therefore, extraction of natural compounds having specific bioactivies and/or medicinal properties from plants is an important application of natural product research.

Due to the unique ecosystem, there are many valuable tree species endemic to Taiwan. In the past few years, we have studied the relationship between wood properties and chemical constituents of endemic trees in Taiwan. In previous studies, we found that *Taiwania cryptomerioides*, *Cinnamomum osmophloeum*, and C*ryptomeria japonica* possess significant antifungal and antitermitic activity (Chang et al., 1998, 1999, 2001a,b; Chang and Cheng, 2002; Cheng and Chang, 2002).

Calocedrus formosana Florin (*Cupressaceae*) is an indigenous tree that grows at elevations of 800–1500 m in the northern part of the central mountain region of Taiwan and is one of the five useful conifer trees in Taiwan. *Calocedrus* is named for its beauty and resin. The wood is pale yellow in color. The physical–chemical properties of the timbers of this plant are recognized for their decay resistance, durability, and an incense-like smell. Its essential oil, of which the acidic constituents (shonanic acid) in particular, was investigated in 1932 by Ichikawa (Lo and Lin, 1956). More than 50 compounds have been isolated including monoterpenes, diterpenes, lignans, and steroids (Lin et al., 1956; Cheng et al., 1961; Fang et al., 1985, 1987, 1989a,b). *C. formosana* leaves have been found to be a suitable cell culture material for hinokitiol (a strong antimicrobial compound) and have the highest hinokitiol production among tested members of the Cupressaceae family (Mikage et al., 1988; Ono et al., 1998). However, there has been little research into the relationship between its wood properties and various extractives. For this reason, the essential oil of *C. formosana* leaves was distilled and its constituents analyzed by GC-MS. The antitermitic activities of the essential oil and isolated constituents against the termite *Coptotermes formosanus* were investigated using direct contact application. In addition, the antifungal activities of these constituents against four wood-rot fungi—*Laetiporus sulphureus*, *Pycnoporus coccineus*, *Lenzites betulina*, and *Trametes versicolor*—were also examined.

METHODS AND MATERIALS

Termite. Coptotermes formosanus Shiraki, was collected from Tainan in southern Taiwan. The colony was reared in an incubator at 26.5°C and 80% RH for more than 1 year. Water and newspapers were used as food sources.

Fungi. Three white-rot fungi [*Lenzites betulina* (CCRC 35296), *Pycnoporus coccineus*, and *Trametes versicolor* (CCRC 35253)] and one brown-rot fungus [*Laetiporus sulphureus* (CCRC 35305)], were used in these experiments. *L. betulina*, *T. versicolor*, and *L. sulphureus* were obtained from the culture collection and research center of the Food Industry Research and Development Institute in Taiwan. *P. coccineus* was a gift provided by Dr. Tun-Tschu Chang (Taiwan Forestry Research Institute).

Essential Oil Distillation. Leaves of 41-year-old *C. formosana* were collected from the Experimental Forest of the National Taiwan University in central Taiwan. Leaf essential oils were extracted by water distillation (8 hr).

Gas Chromatography–Mass Spectrometry (GC-MS) Analysis. The chemical composition of the essential oil was analyzed using GC-MS. An Agilent Technologies HP 5973N mass selective detector in the electron impact (EI) ionization mode (70 eV) was used in conjunction with a Hewlett-Packard 6890 gas chromatograph. Leaf oil constituents were separated under the following conditions: capillary column, HP-1MS (30 m $\times$ 0.25 mm; film thickness 0.25 μm); temperature program, 50°C (held for 5 min) raised to 120°C at a rate of 2°C/min and from 120°C to 220°C at a rate of 5°C/min held for 5 min; injector temperature, 270°C; and carrier gas, helium, at a flow rate of 1 ml/min. Split ratio was 1:10. Identification of the components of *C. formosana* leaf oil was confirmed by comparison with standards as well as by spiking. The quantity of compounds was obtained by integrating the peak area of the spectrograms.

Essential Oil Constituents. The following essential oil constituents were purchased from Acros (Belgium): α-pinene, β-pinene, β-myrcene, limonene, β-caryophyllene, and caryophyllene oxide. α-Cadinol and T-muurolol were isolated from leaf essential oil.

Antitermitic Activity. The no-choice bioassay method of Kang et al. (1990) was used to evaluate antitermitic activity. Samples of 10, 25, and 50 mg of leaf essential oil as well as 1 and 5 mg of each individual compound dissolved in 600 μl of acetone were applied to 1 g filter paper samples (Whatman #3, 8.5 cm in diam). A piece of filter paper treated with solvent only was used as control. After air-drying at room temperature, 50 active termites (45 workers and 5 soldiers) above the third instar were placed onto each filter paper impregnated with the test materials in a Petri dish (9 cm diam $\times$ 1.5 cm height). The test dishes with covers were then placed into an incubator maintained at 26.5°C and 80% RH. A few drops of water were periodically added to the bottom edge of each Petri dish. Three replicates were prepared for each test sample, and the mortality of the termites was counted daily for 14 d.

Antifungal Assay. The method of Chang et al. (1999) was employed for antifungal evaluation of both the essential oil and its main constituents, which were tested at 1000, 200, 100, and 50 μg/ml concentrations against *L. betulina*, *P. coccineus*, *T. versicolor*, and *L. sulphureus* in 9 cm Petri dishes. Antifungal assays were performed three times. After fungal mycelia reached the edges of

control plates (without adding essential oils or compounds) by incubating at 26°C for ca. 10 d, the antifungal index was calculated as follows: Antifungal index (%) = (1−Da/Db) × 100 where, Da= the diameter of growth zone in the experimental plate (cm), Db= the diameter of growth zone in the control plate (cm).

Statistical Analyses. All results were obtained from three independent experiments and expressed as mean ± SD. Significant differences ($P < 0.05$) were determined by using the Scheffe test.

RESULTS AND DISCUSSION

Yields and Chemical Constituents of Essential Oil. The leaf essential oil of *C. formosana* was obtained in a yield of 0.3% (or 3.4 ml/kg) dry weight. GC-MS analysis of the essential oil is shown in Table 1, where components are listed in order of their elution from the HP-1MS column. Nineteen constituents, accounting for more than 94.0% of the total oil composition, were identified. Nine monoterpenes (78.0%) and ten sesquiterpenes (16.5%) were identified in the essential oil. The main components of the leaf essential oil were α-pinene (44.2%),

TABLE 1. CONSTITUENTS OF ESSENTIAL OIL FROM *Calocedrus formosana* LEAVES

Compounds	R_t (min)[a]	Formula	% RA[b]
Tricyclene	6.39	$C_{10}H_{16}$	0.25
α-Pinene	7.29	$C_{10}H_{16}$	44.23
Camphene	7.86	$C_{10}H_{16}$	0.42
β-Phellandrene	9.52	$C_{10}H_{16}$	0.39
β-Pinene	9.64	$C_{10}H_{16}$	1.20
β-Myrcene	11.09	$C_{10}H_{16}$	8.92
Limonene	13.58	$C_{10}H_{16}$	21.57
4-Carene	17.71	$C_{10}H_{16}$	0.55
4-Terpineol	23.47	$C_{10}H_{18}O$	0.48
β-Caryophyllene	40.09	$C_{15}H_{24}$	8.23
α-Caryophyllene	41.83	$C_{15}H_{24}$	0.82
α-Cadinene	44.06	$C_{15}H_{24}$	0.30
γ-Cadinene	44.48	$C_{15}H_{24}$	0.24
δ-Cadinene	44.91	$C_{15}H_{24}$	0.87
Elemol	45.61	$C_{15}H_{26}O$	0.68
Caryophyllene oxide	46.56	$C_{15}H_{24}O$	2.44
γ-Eudesmol	48.22	$C_{15}H_{26}O$	0.25
T-Muurolol	48.48	$C_{15}H_{26}O$	1.09
α-Cadinol	48.81	$C_{15}H_{26}O$	1.56

[a] R_t: Retention time.
[b] RA: Relative area (peak area relative to total peak area).

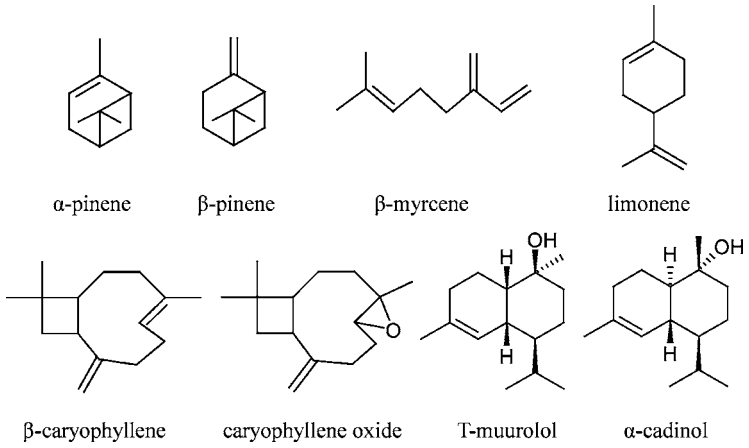

FIG. 1. Structures of terpenoids from *Calocedrus formosana* leaf essential oil.

limonene (21.6%), β-myrcene (8.9%), β-caryophyllene (8.2%), caryophyllene oxide (2.4%), α-cadinol (1.6%), β-pinene (1.2%), and T-muurolol (1.1%). The chemical structures of these compounds are shown in Figure 1.

Antitermitic Activity. The antitermitic activity of *C. formosana* essential oil and various isolated constituents are shown in Figure 2. At a dosage of 10 mg/g, the leaf essential oil killed 26.7% of the termites after 14 d. Termite mortality increased to 76.7% when dosage was increased to 50 mg/g. The LC_{50} value of leaf essential oil against *C. formosanus* was 27.6 mg/g.

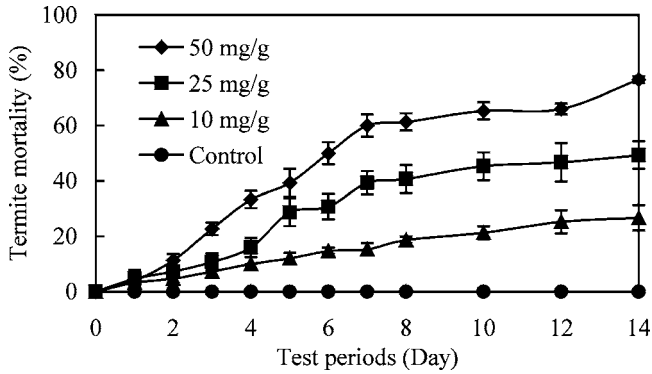

FIG. 2. Antitermitic activity of leaf essential oil from *Calocedrus formosana* against *Coptotermes formosanus*. Means ($N = 3$) using 50 termites per replicate.

TABLE 2. ANTITERMITIC ACTIVITY OF EIGHT ESSENTIAL OIL CONSTITUENTS FROM *Calocedrus formosana* LEAVES AT 1 mg/g DOSAGE

Compounds	Termite mortality[a] (%)	
	7 d	14 d
Limonene	1.3 ± 2.3c	1.3 ± 2.3e
β-Caryophyllene	19.3 ± 2.3a	25.3 ± 2.3a
α-Pinene	2.7 ± 1.2c	5.3 ± 2.3c,d,e
β-Pinene	4.0 ± 2.0c	7.3 ± 3.1c,d,e
β-Myrcene	7.3 ± 3.1b,c	11.3 ± 4.6b,c,d
Caryophyllene oxide	13.3 ± 4.6a,b	17.3 ± 1.2a,b
T-Muurolol	2.0 ± 0.0c	2.0 ± 0.0d,e
α-Cadinol	5.3 ± 3.1b,c	14.0 ± 4.0b,c
Control	0.0 ± 0.0e	0.0 ± 0.0e

[a] Means ($N = 3$) using 50 termites per replicate. Numbers followed by different letters (a–e) are significantly different at the level of $P < 0.05$ according to the Scheffe test.

Antitermitic Activity of Isolated Constituents. To understand the relationship between the main constituents of *C. formosana* essential oil and antitermitic activity, eight constituents were tested for antitermitic activity. Table 2 shows the activity of these constituents at a dose of 1 mg/g. β-Caryophyllene (19.3%) and caryophyllene oxide (13.3%) caused the highest termite mortality after 7 d, followed by β-myrcene (7.3%), α-cadinol (5.3%), β-pinene (4.0%), α-pinene (2.7%), T-muurolol (2.0%), and limonene (1.3%). When the test was extended to 14 d, termite mortality increased slightly (Table 2).

The antitermitic activities of eight isolated oil constituents at 5 mg/g dosages are presented in Figure 3. The order of antitermitic activity was T-muurolol, followed by β-caryophyllene, caryophyllene oxide, α-cadinol, β-myrcene, β-pinene, α-pinene, and then limonene. The respective termite mortalities (at 5 mg/g dosage after 14 d) were 100.0%, 44.0%, 35.3%, 30.0%, 18.7%, 14.0%, 10.7%, and 8.0%. These results agree with those of Ohtani et al. (1997), who also reported the antitermitic activity of α-cadinol and T-muurolol isolated from *Chamaecyparis obtusa* heartwood.

Antifungal Activity of Essential Oil. Figure 4 shows the antifungal index of *C. formosana* leaf essential oil. The constituents were effective in reducing the growth of *L. betulina*, *P. coccineus, T. versicolor,* and *L. sulphureus* at 1000 μg/ml compared with the untreated control. The antifungal indices of the leaf essential oil were 18.9, 67.7, 10.9, and 27.6% against *L. betulina*, *P. coccineus*, *T. versicolor,* and *L. sulphureus*, respectively.

Antifungal Activity of Isolated Constituents. The antifungal indices of the eight compounds at a concentration of 100 μg/ml against the four fungi are

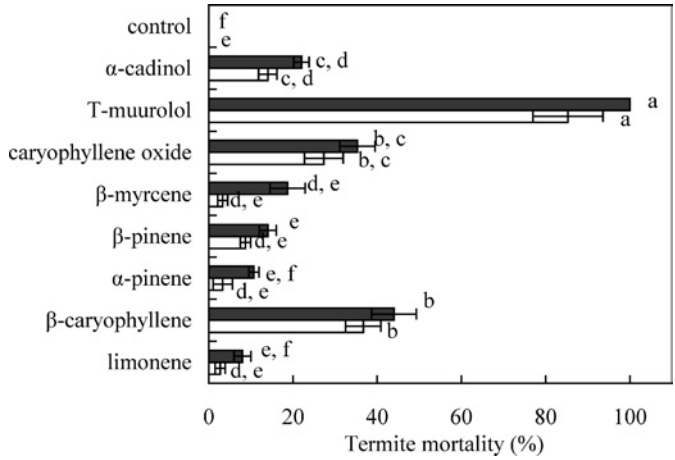

FIG. 3. Antitermitic activity of eight constituents from *Calocedrus formosana* leaf essential oil at 5 mg/g dosages against *Coptotermes formosanus* (white bar, after 7 d; black bar, after 14 d). Means ($N = 3$) using 50 termites per replicate. Numbers followed by different letters (a–f) are significantly different at the level of $P < 0.05$ according to the Scheffe test.

presented in Table 3. The brown-rot fungus was more sensitive to the compounds than the white-rot fungi. In addition, two sesquiterpenes (α-cadinol and T-muurolol) were more effective against the four assay fungi than the monoterpenes. The order of antifungal indices of the eight compounds for *L. sulphureus* was α-cadinol > T-muurolol > caryophyllene oxide > β-caryophyllene > β-myrcene

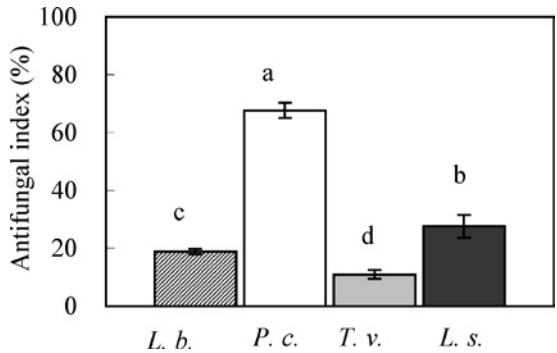

FIG. 4. Antifungal activity of leaf essential oil from *Calocedrus formosana* against fungi (at 1000 μg/ml). Each experiment was performed $\times 3$ and the data averaged ($N = 3$). Numbers followed by different letters (a–d) are significantly different at the level of $P < 0.05$ according to the Scheffe test.

TABLE 3. ANTIFUNGAL ACTIVITY OF ESSENTIAL OIL CONSTITUENTS FROM *Calocedrus formosana* LEAVES AT 100 μg/ml[a]

Constituents	*L. betulina*	*P. coccineus*	*T. versicolor*	*L. sulphureus*
Limonene	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c	21.7 ± 1.2d, e
α-Pinene	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c	19.0 ± 2.6e, f
β-Pinene	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c	17.3 ± 0.8f
β-Myrcene	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c	20.4 ± 3.2e, f
β-Caryophyllene	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c	24.8 ± 2.4d
Caryophyllene oxide	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0c	32.3 ± 2.0c
T-Muurolol	48.0 ± 0.0b	38.1 ± 1.0b	48.0 ± 5.8b	82.0 ± 0.0b
α-Cadinol	100.0 ± 0.0a	65.1 ± 1.4a	100.0 ± 0.0a	100.0 ± 0.0a

[a]Each experiment was performed three times, and the data averaged ($N = 3$). Numbers followed by different letters (a–f) are significantly different at the level of $P < 0.05$ according to the Scheffe test.

> α-pinene > limonene > β-pinene. Among them, α-cadinol and T-muurolol exhibited a higher antifungal activity.

The antifungal effectiveness of α-cadinol and T-muurolol were examined in greater detail. Figure 5 shows the antifungal indices of α-cadinol at serial concentrations. It appears that α-cadinol inhibited completely the growth of *L. betulina, P. coccineus, T. versicolor,* and *L. sulphureus* at a level as low as 200 μg/ml.

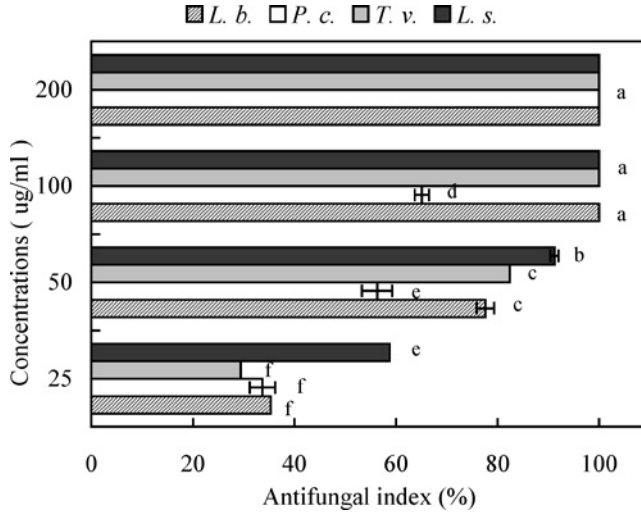

FIG. 5. Antifungal activity of α-cadinol at serial concentrations against fungi. Each experiment was performed $\times 3$ and the data averaged ($N = 3$). Numbers followed by different letters (a–f) are significantly different at the level of $P < 0.05$ according to the Scheffe test.

TABLE 4. MEDIAN LETHAL CONCENTRATION (LC_{50}, μg/ml) OF TWO MAIN CONSTITUENTS FROM THE ESSENTIAL OIL OF *Calocedrus formosana* LEAVES AGAINST FUNGI

Constituents	*L. betulina*	*P. coccineus*	*T. versicolor*	*L. sulphureus*
α-Cadinol	28.6	108.4	30.4	9.9
T-Muurolol	93.3	74.1	81.0	57.3

Similar results were obtained with T-muurolol. Table 4 shows the LC_{50} values of these compounds. It is noteworthy that α-cadinol exhibited the highest antifungal index for *L. betulina*, *P. coccineus*, *T. versicolor,* and *L. sulphureus*, followed by T-muurolol. The LC_{50} values of α-cadinol against *L. betulina*, *P. coccineus*, *T. versicolor*, and *L. sulphureus* are 28.6, 108.4, 30.4 and 9.9 μg/ml, respectively. Further, the LC_{50} values of T-muurolol against four fungi are all below 93.3 μg/ml. These results demonstrate that α-cadinol and T-muurolol showed the significant growth inhibitory effect on all test fungi. In our previous studies on the antifungal performance of *T. cryptomerioides* extractives, α-cadinol exhibited the highest antifungal activity for both *T. versicolor* and *L. sulphureus*, followed by T-cadinol, T-muurolol, ferruginol, and taiwanin C. There have been several reports that α-cadinol exhibits significant effectiveness in durable tree species against fungi or termites (Kondo and Imamura, 1986; Kinjo et al., 1988; Chang et al., 1998, 1999, 2000). The antifungal activity of leaf essential oil isolated from *C. formosana* was attributed to α-cadinol and T-muurolol, or their synergistic effect.

In addition, Table 4 also showed that the antifungal activity of two compounds were higher against brown-rot fungi than against white-rot fungi. Brown- and white-rot fungi decay wood by distinctly different mechanisms. The degradation of wood by white-rot fungi is carried out by enzymes such as cellulase or laccase. The initial stages of brown-rot fungal decay involve oxidative degradation (Highley and Dashek, 1998). DPPH is a stable radical that has been commonly used to evaluate the antioxidant activity of plant and microbial extracts (Gyamfi et al., 1999; Chang et al., 2001c; Wang et al., 2001). Results from our preliminary study showed that both α-cadinol and T-muurolol have inhibitory activity against the DPPH radical (data not shown here), suggesting that they possess some kind of antioxidant activity to prevent the deterioration induced by brown-rot fungi. The significance of antioxidant activities for the cadinane compounds is under investigation and will be addressed in the near future.

In this study, we investigated antitermitic and antifungal activities of leaf essential oil from *C. formosana* against termite and wood decay fungi. Antitermitic tests demonstrated that *C. formosana* leaf essential oil exhibited antitermitic activity. The LC_{50} value of leaf essential oil against *C. formosanus* was 27.6 mg/g after 14 d. Major constituents of *C. formosana* leaf essential oil were identified by

GC-MS. According to antitermitic activity, T-muurolol showed 100% mortality at 5 mg/g after 14 d. Its antitermitic effectiveness is much higher than that using *C. formosana* leaf essential oil. In addition, comparisons of the antifungal effectiveness of these compounds revealed that, among the eight compounds tested, α-cadinol, T-muurolol, and caryophyllene oxide possessed stronger antifungal activity. α-Cadinol exhibited the highest antifungal index for *L. betulina, T. versicolor*, and *L. sulphureus*, it inhibited completely the growth of fungi at levels as low as 100 μg/ml. These results show that α-cadinol and T-muurolol are potential compounds for the development of fungicides or termiticides in the near future.

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BIOACTIVITY OF LIPOPHILIC METABOLITES FROM GLANDULAR TRICHOMES OF *Medicago sativa* AGAINST THE POTATO LEAFHOPPER

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Abstract—*Medicago sativa* cv. G98A is highly resistant to the potato leafhopper, *Empoasca fabae*. Glandular trichome extracts from G98A were fractionated using flash chromatography and tested for settling deterrency against the potato leafhopper. A fraction of intermediate polarity exhibited strong, dose-dependent deterrency when applied to the surface of an artificial diet sachet. Deterrency was not detected, however, when the fraction was applied to the internal surface of the sachet membrane (i.e., when contact was limited to only the leafhoppers' stylets). Major components of the highly deterrent fraction, determined by gas chromatography – mass spectrometry, were a homologous series of fatty acid

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amides $C_nH_{2n+1}NO$ ($n = 19$–23) and trace components were 12:0, 13:0, 14:0, 15:0, 16:0, 17:0, 18:0, and possibly 18:1 free fatty acids. Deterrency declined slightly, but was still strong, after fatty acids were removed from crude extracts. When the crude extracts were separated further, a fraction containing only the fatty acid amides was also deterrent. Activity increased when this fraction was supplemented with authentic (C12:0 through C18:0, and C18:1) free fatty acids. However, the authentic free fatty acids were not deterrent when tested without the lipophilic amides. Fatty acid amides and free fatty acids in trichomes of *M. sativa* G98A may synergize and together function in deterring settling by the potato leafhopper.

Key Words—Fatty acid amides, potato leafhopper, *Empoasca fabae*, alfalfa, *Medicago sativa*, host selection, glandular trichomes.

INTRODUCTION

The potato leafhopper, *Empoasca fabae* (Harris), is a generalist herbivore known to feed on over 200 host plants (Lamp et al., 1995). This insect is also considered to be the most significant pest of alfalfa, *Medicago sativa* L., in the midwestern and eastern United States. Cultivars of glandular-haired alfalfa expressing resistance to the potato leafhopper are currently commercially available. Consequently, *M. sativa* glandular trichomes have been the focus of recent studies, because of their presumed role in providing protection against the potato leafhopper (Hogg and McCaslin, 1994; Elden and McCaslin, 1997; Ranger and Hower, 2001a,b, 2002; Shockley et al., 2002; Shockley and Backus, 2002).

Under field conditions and high leafhopper densities, resistant cultivars exhibit favorable growth characteristics, such as reduced leaf yellowing (i.e., hopperburn) and plant stunting (Lefko et al., 2000a,b; Sulc et al., 2001). Glandular-haired cultivars also have the ability to reduce field populations of potato leafhopper nymphs (Sulc et al., 2001). In addition, laboratory studies demonstrated that potato leafhopper development (Hogg and McCaslin, 1994), survivorship (Hogg and McCaslin, 1994; Elden and McCaslin, 1997; Ranger and Hower, 2001b; Shockley et al., 2002), and host selection (Ranger and Hower, 2002; Shockley and Backus, 2002) were affected by the presence of *M. sativa* glandular trichomes.

Principally, the trichome-based resistance mechanism of glandular-haired *M. sativa* is associated with secretion of biologically active metabolites by erect and/or procumbent glandular trichome types (Hogg and McCaslin, 1994; Elden and McCaslin, 1997; Ranger and Hower 2001a,b, 2002; Shockley et al., 2002). For instance, crude glandular trichome extracts from the resistant *M. sativa* genotype G98A deterred settling by potato leafhopper adults, and settling declined with an increase in extract concentration (Ranger et al., 2004). Fatty acid amides were major components of trichome extracts from *M. sativa* G98A (Ranger et al., 2004). These amides were not present in trichome extracts from less resistant glandular-haired *M. sativa* cv. G98C or from susceptible, nonglandular-haired *M. sativa*

cv. Ranger. Fatty acid amides are widespread in nature (Hannun et al., 1996) and have been isolated from a number of plants, grasses, and algae (Parmar et al., 1997; Faulds and Williamson, 1999; Kawasaki et al., 1998; Dembitsky et al., 2000). Here, we describe the fractionation and bioassay of major and some minor trichome components from *M. sativa* G98A.

METHODS AND MATERIALS

Insects and Plants. A colony of potato leafhoppers was maintained according to Hunter and Backus (1989). Insects were reared on greenhouse-grown fava beans *Vicia faba* (cv. "Windsor") using an environmental growth chamber (25 ± 2°C; 16:8 hr L:D). Cuttings (i.e., ramets) of resistant *M. sativa* genotype G98A were provided by Cal/West Seeds (West Salem, WI). Plants were vegetatively propagated and grown under greenhouse conditions. Metal halide lamps (400-W high-pressure sodium, were used to supplement the natural lighting. Experimental plants were about 4 mo old and harvested three times prior to use. In addition, extracts used in experiments were obtained from plants allowed to grow for about 25 days since last cutting.

Trichome Extraction and Fractionation. Trichomes were isolated from 150 g (fresh weight) of *M. sativa* G98A stem sections according to a modified protocol of Yerger et al. (1992) and described in detail by Ranger et al. (2004). In short, entire stems were harvested from 11:00 A.M. to 3:00 P.M. and immediately cut into 3-cm sections. About 1–2 g was transferred into a test tube and lowered into a Dewar flask containing liquid nitrogen (N_2). After submersion, the test tube was raised out of N_2 and vortexed for 3–5 sec, resulting in the freed trichomes adhering to the test tube walls. Isolated trichomes were rinsed and soaked in ethanol for 24 hr, along with 10 g Na_2SO_4 (as an overnight drying agent). Extracts were filtered through glass fiber filter paper (G6, Fisher Scientific, Pittsburgh, PA) and evaporated under reduced pressure using a rotary evaporator. Residues (crude weight: 17 mg) were redissolved in 2 ml of methylene chloride for fractionation.

Crude trichome extracts from *M. sativa* G98A were fractionated using flash chromatography (Still et al., 1978) (0.04–0.063 mm, 230–400 silica gel) with elution with methylene chloride containing increasing concentrations of ethanol. Four fractions were collected using the following elution concentrations: fraction 1 (F1) 100% methylene chloride; fraction 2 (F2) 3% ethanol in methylene chloride; fraction 3 (F3) 6% ethanol in methylene chloride; and fraction 4 (F4) 25–100% ethanol. Fractions were evaporated to dryness and residues of each fraction were redissolved in methylene chloride for gas chromatography – mass spectrometry (GC–MS) analysis or 2 ml of acetone for bioassay.

Preparation of Neutral and Acidic Extracts. The role of neutral and acidic compounds in deterring leafhopper settling was assessed by first isolating

trichomes from 150 g of G98A stem sections and extracting with ethanol. Crude extracts (with acidic compounds present) were concentrated to dryness using a rotary evaporator, and residues (crude weight: 17 mg) were redissolved in 2 ml of acetone for bioassay.

After testing crude trichome extracts with acidic compounds present, extracts were next concentrated to dryness and redissolved in 2 ml of methylene chloride. Acidic compounds were removed from the extracts by eluting through a column of Alumina (basic Grade I, Merck Co.) using 6% ethanol in methylene chloride. The resulting neutral eluants were concentrated to dryness by using a rotary evaporator, and residues (3.5 mg) were redissolved in 2 ml of acetone for bioassay.

After bioassay, the acid-free extracts were concentrated to dryness, redissolved in 2 ml of methylene chloride, and fractionated with flash chromatography (using the aforementioned procedures). The neutral fraction collected with 6% ethanol in methylene chloride was concentrated to dryness and residues (2.9 mg) were redissolved in 2 ml of acetone for bioassay.

A blend of authentic fatty acids (C12:0 through C18:0, and C18:1) was added to the neutral fraction. The mixture was added to the neutral fraction to represent, on the basis of GC–MS analysis, 10% of the total concentration of lipophilic amides present in the sample (2.9 mg). The total percent contribution of each free fatty acid to the stock mixture were as follows: 12:0 (5%), 13:0 (5%), 14:0 (10%), 15:0 (5%), 16:0 (54%), 17:0 (5%), 18:0 (4%), and 18:1 (12%). Finally, to test the free fatty acids alone, the same fatty acid blend was added to an acetone stock at the same concentration as was added to the neutral fraction (i.e., 10% of the amides).

Insect Acclimation for Bioassays. Prior to each bioassay, <5 day-old adult females were selected from the laboratory fava bean colony and transferred to a cage containing two mature *M. sativa* Ranger plants for a 24 hr period of conditioning. Insects were then transferred to a sachet containing artificial diet for an additional 24 hr of acclimation. Acclimation sachets were based on a design by Habibi et al. (1993) and described in detail by Ranger et al. (2004). In short, acclimation sachets were prepared by stretching Parafilm® over a disc of solidified artificial diet (approximately 8.0-cm diameter, 1.0 cm high). Sachets were placed Parafilm® side up in the bottom of petri dish (8.5-cm diameter), and a petri dish lid was used to contain the leafhoppers. Artificial diet consisted of an aqueous solution of 5% (wt:vol) sucrose and 4% (wt:vol) low-melt agarose (Sea Plaque Agarose, FMC Inc., Rockland, ME).

Two-Choice Bioassays of Fractionated Extracts. Fractionated trichome extracts were bioassayed using a feeding sachet based on a design by Habibi et al. (1993) and described in detail by Ranger et al. (2004). In short, sachets were prepared by pipetting artificial diet into a single plastic gasket (1×1 cm^2) positioned on a microscope cover slip. Parafilm® was stretched over the gasket and trimmed

back to within the gasket edges. Completed sachets were stored at 1°C for 24 hr prior to their use in bioassays.

A micropipette was used to apply aliquots from stock solutions to the exposed Parafilm® surface of the sachet. The acetone was allowed to evaporate from the sachet surface under ambient conditions for about 25 min. Each of the four fractions were tested at concentrations representing 7.5, 3.75, and 1.88% of the initial stock solution. In all experiments, control sachets were prepared by applying aliquots from an acetone stock (using the corresponding volume for a particular comparison).

After fractions were applied to sachets, two-choice comparisons were made by placing a treated and control sachet at opposing regions within test arenas, which consisted of a clear plastic tube (7.0 cm diameter, and 3.5 cm in high) positioned upright in a Petri dish lid. Once sachets were situated, a Petri dish lid was used to seal the test arena, and 10 acclimated leafhoppers were aspirated into each arena. Test arenas were arranged in a completely randomized design in an aluminum tray under constant fluorescent light, and water was added to the bottom of the tray to prevent desiccation. After 60 min of acclimation, the number of insects settling/feeding on a particular diet surface was recorded at 15 min intervals over the next 350 min.

Numbers of leafhoppers settling on each sachet were converted into proportions and arcsine square root-transformed. Data were analyzed with the SAS general linear model (GLM) procedure (SAS Institute, 1985) using a repeated measures split plot analysis of variance (ANOVA). Treatment was used as the main plot effect in the linear statistical model for comparisons of settling. The subplot contained the effects of Time and Treatment × Time. If a significant Treatment × Time interaction was present, then differences between means for ANOVAs were compared with least significant difference (SAS Institute, 1985).

Site of Activity. To determine the portion of the potato leafhopper's feeding apparatus on which the compounds in F3 act, a modified two-choice bioassay was conducted whereby 1.88% of the 2 ml F3 solution was first applied to the Parafilm® surface and the solvent was allowed to evaporate. The piece of Parafilm® was then inverted before it was laid over the artificial diet, resulting in the treated surface coming in direct contact with the diet. The Parafilm® surface exposed to the labium and the rest of the body was consequently untreated, thereby limiting contact with F3 to only the leafhoppers' stylets. Control sachets were prepared using the corresponding volume of acetone. Treated and control sachets were stored at 1°C for 24 hr, to allow the extract to diffuse partially. After this time, sachets were tested for settling deterrency using the previously described two-choice bioassay. Data were transformed and analyzed using the aforementioned procedures.

Effects of Acid Removal and Addition on Activity. Crude extracts with acidic compounds present and absent were bioassayed at 1.88% of the initial stock

solution, using two-choice design. A neutral fraction with composition similar to F3 was also tested at 1.88% of the initial stock, and also after being supplemented with authentic free fatty acids. The same blend of authentic fatty acids was also tested alone at 1.88% of the stock. In each experiment, numbers of leafhoppers settling on trichome extracts were compared with numbers settling on a solvent control.

GC–MS Analysis. Crude and fractionated extracts redissolved in methylene chloride were analyzed using an Hewlett-Packard 5890 gas chromatograph equipped with a mass spectra detector operating in electron impact (EI) mode (70 eV). A Restek Rtx-1 column (15 m × 0.25 mm × 0.3 μm) or equivalent was used. The injector port was held at 250°C, and the oven was programmed from 100 to 320°C at 10°C/min and held at 320°C for 5 min.

RESULTS

Two-Choice Bioassays of Fractionated Extracts. At 7.5% of the initial stock solution, or 488 μg/cm^2, the third fraction (F3) collected by flash chromatography of *M. sativa* G98A trichome extracts possessed the most significant degree of deterrency in two-choice bioassays ($F = 73.04$; $df = 1$; $P < 0.001$; Figure 1C). This fraction weighed 6.5 mg and represented about 38% of the total material recovered, compared to 5 mg for F1, 2.5 mg for F2, and 3.0 mg for F4. At 7.5% of the initial stock, or 225 μg/cm^2, F4 provided the only other deterrency, but it was not highly active ($F = 5.30$; $df = 1$; $P = 0.044$; Figure 1D). Similarly, the number of leafhoppers settling on F2 was only marginally less than those on control sachets when tested at 188 μg/cm^2 ($F = 4.43$; $df = 1$; $P = 0.059$; Figure 1B). F1 did not significantly ($P > 0.05$) deter leafhopper settling compared against control sachets at 375 μg/cm^2 (Figure 1A). A Treatment × Time interaction was not detected ($P > 0.05$) for any of the fractions tested at 7.5% of the initial stock solution.

F3 was also highly deterrent to leafhopper settling when tested against control sachets at 3.75% of the initial stock solution, or 244 μg/cm^2 ($F = 41.21$; $df = 1$; $P < 0.001$; Figure 2C). A Treatment × Time interaction was detected for F4 at 3.75% of the initial stock solution, or 115 μg/cm^2 ($F = 2.47$; $df = 18$; $P < 0.001$; Figure 2D). Control sachets were not significantly ($P > 0.05$) preferred over F1 at 188 μg/cm^2 (Figure 2A) or F2 at 94 μg/cm^2 (Figure 2B). At 3.75% of the initial stock solution, a Treatment × Time interaction was not detected ($P > 0.05$) for F1, F2, or F3.

Activity slightly declined with a decrease in concentration, but F3 remained highly active at 1.88% of the initial stock solution, or 122 μg/cm^2 ($F = 33.30$; $df = 1$; $P < 0.001$; Figure 3C). F2 at 47 μg/cm^2 did not significantly ($P > 0.05$) affect selection behavior of the potato leafhopper (Figures 3B). A Treatment

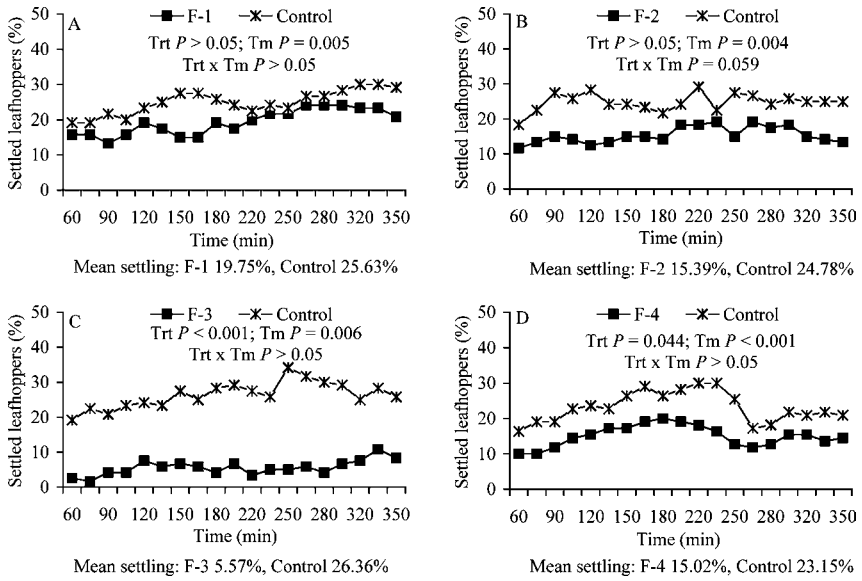

FIG. 1. (A–D). Two-choice bioassay of potato leafhoppers for settling on fractions from *M. sativa* G98A glandular trichomes or a solvent control. Fractions were applied to the surface of an artificial diet sachet at a concentration of 7.5% of the initial stock solution, which represented 375 μg/cm^2 for F1 (A), 188 μg/cm^2 for F2 (B), 488 μg/cm^2 for F3 (C), and 225 μg/cm^2 for F4 (D). $N = 12$ for (A), (B), and (C), and $N = 11$ for (D). Results from repeated measurements split plot ANOVA are provided (see Methods and Materials for details).

$\times$ Time interaction was detected for F4 at 1.88% of the initial stock solution, or 56 μg/cm^2 ($F = 2.22$; $df = 18$; $P = 0.003$; Figure 3D). F1 was significantly preferred to control sachets at 1.88% of the initial stock, or 94 μg/cm^2 ($F = 6.22$; $df = 1$; $P = 0.03$; Figure 3A). At 1.88% of the initial stock solution, a Treatment $\times$ Time interaction was not detected ($P > 0.05$) for F1, F2, or F3.

Site of Activity. When F3 was applied at 1.88% of the initial stock solution, or 122 μg/cm^2, to the Parafilm® sachet's inner surface, the number of insects settling on treated sachets did not differ ($P > 0.05$) from control sachets (Figure 4). Therefore, leafhoppers were not deterred from settling when contact with F3 was limited only to their stylets.

GC–MS Analysis of Trichome Fractions. Major components of the highly active F3 were a homologous series of fatty acid amides $C_nH_{2n+1}NO$ ($n =$ 19–23), which have been previously characterized by electron impact and fast atom bombardment mass spectrometry (Ranger et al., 2004). The compounds are *N*-(3-methylbutyl)amides of straight chain C14:0 through C18:0 fatty acids, as

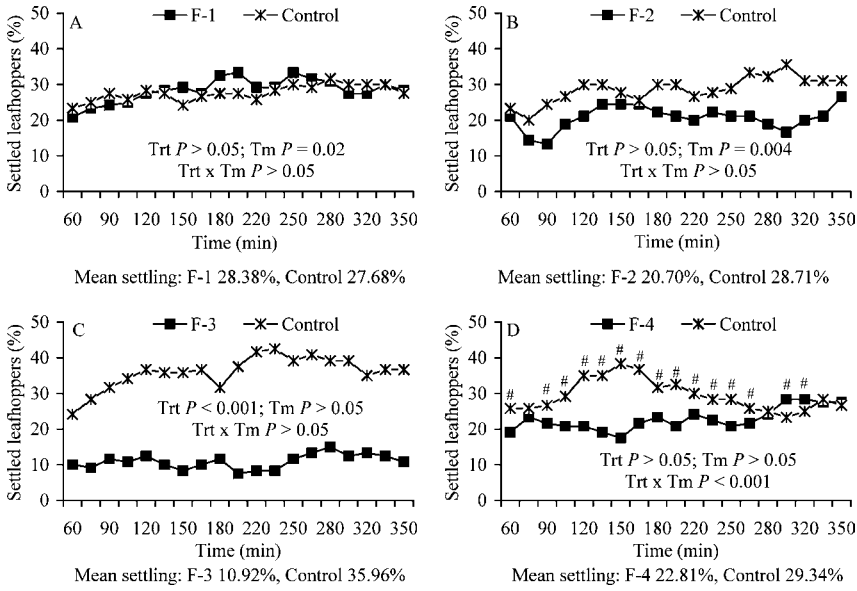

FIG. 2. (A–D). Two-choice bioassay of potato leafhoppers for settling on fractions from *M. sativa* G98A glandular trichomes or a solvent control. Fractions were applied to the surface of an artificial diet sachet at a concentration of 3.75% of the initial stock solution, which represented 188 μg/cm^2 for F1 (A), 94 μg/cm^2 for F2 (B), 244 μg/cm^2 for F3 (C), and 115 μg/cm^2 for F4 (D). Results from repeated measurements split plot ANOVA are provided (see Methods and Materials for details). Since a significant Treatment $\times$ Time interaction was associated with (D), any difference between the percentage of settled leafhoppers greater than an LSD value of 2.5% are significantly different ($P > 0.05$) and are distinguished by #. $N = 12$ for (A) and (D) and $N = 9$ for (B) and (C).

determined by comparison of mass spectra and retention times with those of synthetic representatives (Ranger, unpublished data). The fatty acid amides were in greatest concentration in F3, which was collected by eluting with 6% ethanol in methylene chloride. Trace components in the deterrent F3 were a homologous series of saturated free fatty acids $C_nH_{2n}O_2$ ($n = 12$–18), and an unsaturated C18 acid, likely oleic acid. Fatty acids and some of the amides were also present in lower concentration in the less active F4 fraction (Figure 1D). Similarly, trace amounts of the amides and fatty acids were present in F2, but this fraction was not deterrent at the concentrations tested.

Major components of F1, which was preferred to control sachets at 1.88% of the initial stock, or 93.75 μg/cm^2 (Figure 3A), were hydrocarbons and/or related alcohols, collected using 100% methylene chloride. Fatty acid amides and free fatty acids were not present in F1.

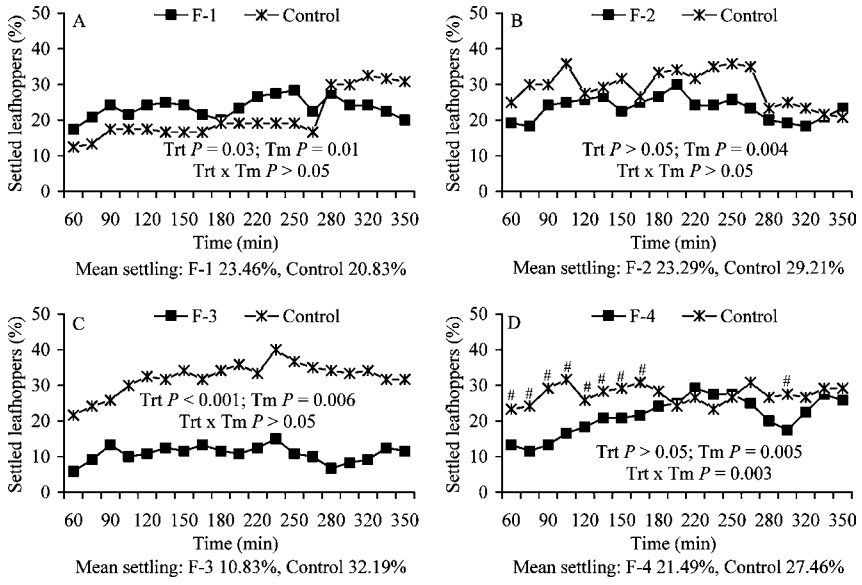

FIG. 3. (A–D). Two-choice bioassay of potato leafhoppers for settling on fractions from *M. sativa* G98A glandular trichomes or a solvent control. Fractions were applied to the surface of an artificial diet sachet at a concentration of 1.88% of the initial stock solution, which represented 94 μg/cm^2 for F1 (A), 47 μg/cm^2 for F2 (B), 122 μg/cm^2 for F3 (C), and 56 μg/cm^2 for F4 (D). Results from repeated measurements split plot ANOVA are provided (see Methods and Materials for details). Since a significant Treatment × Time interaction was associated with (D), any difference between the percentage of settled leafhoppers greater than an LSD value of 7.8% for (D) are significantly different ($P > 0.05$) and are distinguished by #. $N = 12$ for each comparison.

Effects of Acid Removal and Addition on Activity. Control sachets were significantly preferred to sachets treated with crude extracts ($F = 70.07$; $df = 1$; $P < 0.001$; Figure 5A). GC–MS confirmed the extracts contained lipophilic amides, free fatty acids, and linear hydrocarbons (and/or related alcohols).

The neutral extracts, when tested at 1.88% of the stock solution, or 66 μg/cm^2, were less deterrent ($F = 21.15$; $df = 1$; $P < 0.001$; Figure 5B). GC–MS confirmed the absence of free fatty acids, and the presence of fatty acid amides and hydrocarbons (and/or related alcohols) in the neutral extracts. At 1.88% of the initial stock, or 54 μg/cm^2, the amide-containing fraction remained deterrent against leafhopper settling ($F = 30.11$; $df = 1$; $P < 0.001$; Figure 5C). Activity did not appear to decline with loss of the hydrocarbons and/or related alcohols (compare Figure 5B and C).

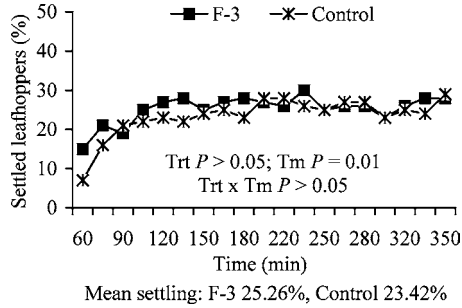

FIG. 4. Deterrency of fraction F3 from *M. sativa* G98A trichome extracts when contact was limited to the potato leafhoppers' stylets. Leafhoppers were offered a choice between sachets treated with F3 or a solvent control ($N = 12$). F3 was applied to the surface of an artificial diet sachet at a concentration of 1.88% of the initial stock solution, which represented 122 mg/cm^2. Results from repeated measurements split plot ANOVA are provided (see Methods and Materials for details).

To test for synergism, when free fatty acids were added to the amide-containing fraction and tested at 1.88% of the initial stock, or 59.5 μg/cm^2, the extracts became highly deterrent to leafhopper settling ($F = 115.50$; $df = 1$; $P < 0.001$; Figure 5D). However, at 1.88% of the stock, or 5.45 μg/cm^2, the blend of authentic free fatty acids alone was not deterrent against leafhopper settling ($P > 0.05$; Figure 6).

DISCUSSION

The presence of saturated fatty acid amides predominantly in the highly deterrent F3 suggests that these compounds possess biological activity. Lipophilic amides were the only GC–MS detectable compounds in a deterrent neutral fraction (Figure 5C) after free fatty acids were selectively removed. Fatty acid amides have not been well documented to affect insect behavior, but isobutylamides have long been known to possess insecticidal activities (Elliot et al., 1987). For instance, plant-derived isobutylamides of unsaturated, aliphatic, straight chain C10–C18 acids are toxic to a variety of insects (Jacobson, 1971).

Saturated (C12:0 through C18:0) and possibly unsaturated (18:1) free fatty acids in the deterrent F3 also suggest that these compounds assist in deterring leafhopper settling. Deterrency decreased when fatty acids were removed from crude *M. sativa* G98A trichome extracts (compare Figure 5A and B). While this decline in activity may be explained by partial loss of active compounds after elution through basic Alumina, supplementation of the amide-containing fraction with authentic fatty acids (C12:0–C18:0, and C18:1) resulted in increased

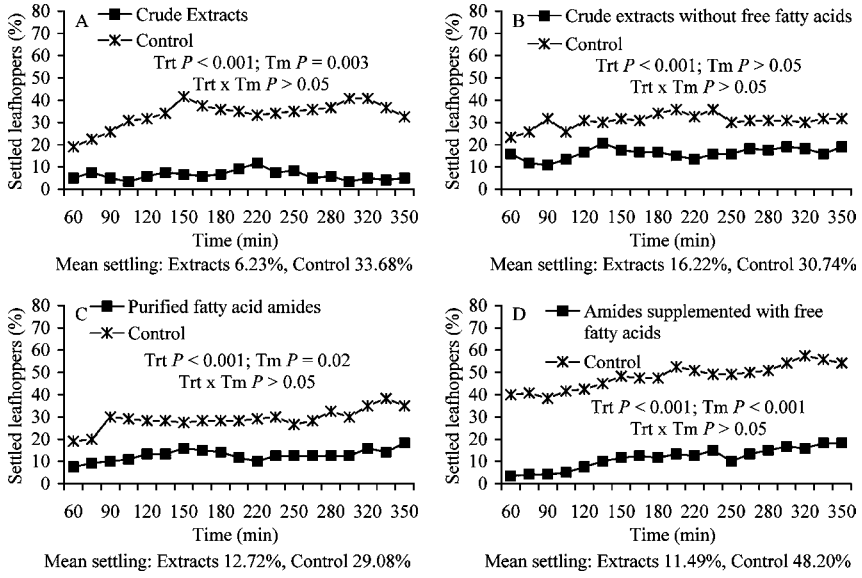

FIG. 5. (A–D). Activity of crude *M. sativa* G98A glandular trichome extracts with acidic compounds present (A) was compared against a solvent control. Acidic compounds were removed from the crude extracts (B) and bioassayed. Next, an amide-containing fraction (C) was isolated from the neutral, crude extracts, and tested for activity. The neutral, amide-containing fraction was supplemented with a mixture of authentic C12:0 through C18:0, and C18:1, free fatty acids (D). All extracts were tested at 1.88% of the initial stock solution, which represented 319, 66, 54, and 59.5 μg/cm^2 for (A), (B), (C), and (D), respectively. $N = 12$ for each comparison. Results from repeated measurements split plot ANOVA are provided (see Methods and Materials for details).

deterrency (Figure 5D). In fact, the degree of deterrency appeared to return to that of the crude extracts (compare Figure 5A and D). However, when tested alone at 5.45 μg/cm^2, the mixture of free fatty acids alone was not deterrent. These results suggest that C12–C18 free fatty acids may function in a synergistic fashion with the lipophilic amides, possibly by serving as a "carrier" for the deterrent amides. Synthetic representatives of the fatty acid amides and free fatty acids from *M. sativa* G98A glandular trichomes are currently being bioassayed to more completely understand the activity and interactions among these compounds. Settling by the green peach aphid, *Myzus persicae* Sulzer, is deterred by C8–C13 fatty acids at concentrations ranging from 1 to 100 μg/cm^2 (Greenway et al., 1978). Furthermore, longer chain free fatty acids (>C16) make up a large proportion of the surface lipids of some plants, but their effect on insect behavior is not well known (Eigenbrode and Espelie, 1995).

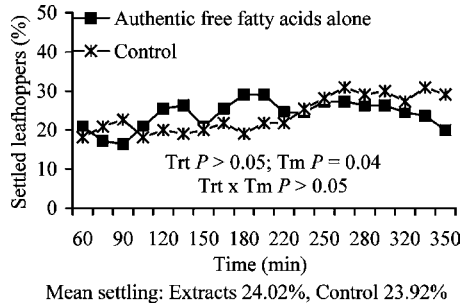

FIG. 6. Two-choice bioassay of potato leafhoppers for settling on artificial diet sachets treated with a mixture of C12:0 through C18:0, and C18:1, free fatty acids or a solvent control. The fatty acid mixture was tested at 1.88% of the initial stock solution, or 5.45 $\mu g/cm^2 (N = 11)$. Results from repeated measurements split plot ANOVA are provided (see Methods and Materials for details).

Since the piercing–sucking potato leafhopper is not ingesting the glandular trichomes, as would a chewing insect, it seems the deterrence of *M. sativa* G98A trichome extracts is a function of contact with the labium or other nonstylet part(s) of the body. The lack of deterrency observed when contact with the highly deterrent F3 was limited to only the leafhoppers' stylets (Figure 4) suggests that uptake to precibarial sensilla or the gut may not be necessary for deterrence. Similarly, sucrose esters of carboxylic acids from glandular trichomes of *Solanum berthaultii* Hawkes were not effective in deterring settling or probing by the green peach aphid when contact was limited to only the stylets (Neal et al., 1990). Chapman (1995) noted that Hemiptera lack chemoreceptors on the stylets, and while they may be present on the labrum and labium, they are used to detect chemicals on the external surface of the host (Backus, 1988; Chapman, 1995). However, it is possible that ingestion of alfalfa trichome metabolites by the potato leafhopper acts in conjunction with external stimuli (affecting the exoskeleton or contact chemoreceptors) to cause deterrence.

When stationary on the stem surface, the potato leafhopper's exoskeleton often comes in direct contact with secretory gland heads of the erect trichome type (Ranger, personal observation). Following contact, the lipophilic trichome metabolites might be absorbed through the cuticle and affect the potato leafhopper's physiology. Compared to the erect glandular trichome, however, the morphology of the procumbent trichome type appears better suited for positioning an exudate in such a manner to encounter the leafhopper's tarsal chemoreceptors. In essence, contact between the leafhopper's tarsi and secretions from the procumbent glandular trichome seems likely because the procumbent trichome is characterized by a bent stalk, which results in the gland head being in close proximity to the surrounding epidermal surface (Kreitner and Sorensen, 1979a; Ranger and

Hower, 2001a). Exudate released from the procumbent trichome then coats the epidermal surface near the collapsed gland head (Kreitner and Sorensen, 1979b; Ranger and Hower 2001a) and has even been observed to adhere to the potato leafhopper's tibia (Ranger and Hower, 2001a). Following contact with the contact chemoreceptors, trichome metabolites such as the fatty acid amides may then be recognized as deterrents. Alternatively, sensitivity of the tarsal chemoreceptors to phagostimulants may be reduced or impaired by the trichome metabolites. Potato leafhopper nymphs clean their tarsi using an excretory droplet significantly more often following contact with glandular trichomes on the resistant *M. sativa* cv. FGplh13 compared to nonglandular trichomes on a susceptible variety (Ranger and Hower, 2002). Such a behavioral response indicates recognition on part of the potato leafhopper to either the adhesive exudate or compounds present within the secretion.

Interestingly, F1 was actually attractive to the leafhopper when tested at 94 μg/cm^2 (Figure 3A). Linear hydrocarbons and/or related alcohols were the main components of this nonpolar fraction and may have been responsible for increased settling. A hydrocarbon fraction of *n*-alkanes (C27, C29, C31, and C33) from broad bean, *Vicia faba* L. stimulated feeding by the pea aphid, *Acyrthosiphon pisum* (Harris) (Klingauf et al., 1971, 1978). Additional studies are also needed to characterize the effects of hydrocarbons from *M. sativa* G98A on host selection by the potato leafhopper.

Overall, fatty acid amides and free fatty acids in glandular trichomes of *M. sativa* G98A appear to play a role in deterring settling by the potato leafhopper. Identification of natural products from glandular-haired *M. sativa* with activity against the potato leafhopper has significant implications for determining the chemical and molecular mechanisms by which resistance operates. The ability to screen for plants expressing high concentrations of deterrent compounds could increase the efficiency by which improved cultivars are selected.

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GENETIC VARIATION IN PRIMARY METABOLITES OF *Pastinaca sativa*; CAN HERBIVORES ACT AS SELECTIVE AGENTS?

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Abstract—Although insect herbivory has been shown to act as a selective agent on plant secondary metabolism, whether primary metabolites contribute to resistance and can respond to selection by herbivores remains untested. In the wild parsnip (*Pastinaca sativa)*, its principal herbivore, *Depressaria pastinacella*, acts as a selective agent on furanocoumarin resistance factors. In this study, we determined whether webworms can, by causing differential reductions in fitness, act as selective agents on parsnip primary metabolites. Estimates of narrow-sense heritabilities were significantly different from zero for C_{18} fatty acids in buds and developing fruits, fructose and sorbitol in buds, fructose, myo-inositol, bergapten, and psoralen in fruits. Wild parsnips protected from webworms by insecticide produced 2.5 times as much seed biomass as unsprayed plants; that webworms accounted for this difference in plant fitness was indicated by a significant negative relationship between reproductive effort and an index of webworm damage. Only a handful of metabolites influenced resistance to webworms; these included osthol, sorbitol, and protein in developing fruits as well as previously documented furanocoumarins. Osthol, a coumarinic compound, enhanced resistance, as did protein content, while sorbitol lowered resistance. Other primary metabolites may affect resistance to webworms, but their effect was context-dependent, that is, their effect depended on concentrations of other metabolites (epistasis). Susceptible plant phenotypes were found to have average chemical compositions. Although there was genetic variation in some of the primary metabolites in parsnips, the epistatic nature of their involvement in resistance and the lack of genetic variation in some suggest that selection on them from webworms will be either inconsistent or ineffective.

Key Words—Nutrients, defense, *Pastinaca*, *Depressaria*, heritability, selection, herbivory, fatty acids, sugars, natural selection.

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INTRODUCTION

That secondary metabolites determine, in part, the suitability of plants to herbivorous insects is established beyond, debate, also largely indisputable is the importance of nutrients in determining suitability of plant tissues for herbivores (Berenbaum, 1995). Effects of nutrient variation on herbivore preference and performance have been well documented (Slansky and Rodriguez, 1987; Simpson and Simpson, 1990; Slansky, 1992). What remains a question, however, is the extent to which insect herbivory can act as a selective agent on the nutrient content of plants. The concept that plant nutrient quality can evolve in response to herbivore selection pressure was articulated early on by both Feeny (1976) and Rhoades and Cates (1976), although theoretical objections (Moran and Hamilton, 1980; Price et al., 1980) were also raised based on the fact that a decrease in the quality or quantity of nutrients could potentially cause greater plant damage because of the ability of some herbivores to compensate for low nutrient levels by increasing ingestion (Slansky and Feeny, 1977). Although the idea that low nutritive quality can evolve as a plant defense has been in the literature for over two decades, it has been the focus of remarkably few studies (Neuvonen and Haukioja, 1984; Lundberg and Astrom, 1990).

As is the case for any trait, in order for plant primary metabolites to change in response to herbivore selection pressure, genetic variation must exist for the production of these primary metabolites. Evidence in support of such variation is almost entirely derived from work on crop plants, in which "nutrient" is defined by human nutritional needs. Genetic variation exists, for example, in unsaturated fatty acid content and composition (Knowles and Mutwakil, 1963; Kinman, 1972; Widstrom and Jellum, 1975; Kondra and Wilson, 1976; Gathman and Bemis, 1983; Brandle et al., 1993), protein concentration (Sullivan and Bliss, 1983; McFerson and Frey, 1991; Caradus, 1992; Hansen et al., 1992; Moser and Frey, 1993) and composition (Axtell, 1981), sugar content and composition (Poehlman, 1979; Bajaj et al., 1980; Simon et al., 1982; Freeman and Simon, 1983; Stommel and Simon, 1989), and vitamin content (Mozafar, 1994).

A second prerequisite for the evolution of plant primary metabolism in response to insect herbivory is the existence of a predictable association between low nutrient quality and plant resistance to insects. Again, evidence exists of such an association, although it derives largely from the crop literature. Resistance to insects has been associated with the ratio of sugar to nitrogen (Maltais and Auclair, 1957), the type and quantity of plant sugars (Clancy, 1992; Zou and Cates, 1994), free amino acid content (Auclair et al., 1957), and amino acid imbalance (Janzen, 1977). Primary metabolites can also mediate resistance indirectly by influencing the toxicity of secondary metabolites (Slansky, 1992); increased protein, for example, can ameliorate the toxicity of trypsin inhibitors and glycoalkaloids, and antioxidant vitamins can counter allelochemical oxygen-dependent phototoxicity

(Aucoin et al., 1990; Green and Berenbaum, 1994). Secondary metabolites can also enhance or potentiate the detrimental impact of low nutrient quality; diet dilution can bring about compensatory feeding, which may cause a herbivore to ingest greater quantities of a toxin (Slansky and Wheeler, 1992).

Evolutionary responses in plant primary metabolism to insect herbivory are most likely to occur when a highly oligophagous herbivore with limited mobility must complete development on a single plant, or even a single plant part (such as a fruit or seed) and, thus, has no opportunity to compensate for nutritional deficiencies by switching host plants. Such herbivores, particularly seed feeders on monocarpic plants, are likely to be important selective agents on their host plants. It is, however, in situations in which herbivores consume seeds or fruits that the conflict between plant and herbivore nutrient needs is most acute; because they do not photosynthesize, developing embryos use plant nutrients as do heterotrophs, such as insects, that eat plants. Seedling performance with respect to growth, germination rate, or competition is often dependent upon the nutrient content of the seed (Rahman and Goodman, 1983; Parrish and Bazzaz, 1985; Varis and George, 1985).

Primary metabolites may well play a role in the interaction between parsnip webworms and wild parsnips. *Depressaria pastinacella* is an oligophagous species that feeds exclusively on the buds, flowers, and green fruits of the monocarpic biennial *Pastinaca sativa* throughout most of its range in North America, where, along with its principal hostplant, it has been introduced from Europe (Hodges, 1974). By feeding on reproductive parts, this insect can greatly affect plant fitness and thereby act as a selective agent on the content and composition of furanocoumarins, secondary metabolites associated with resistance (Berenbaum et al., 1986, 1989; Zangerl and Berenbaum, 1993). Differences in furanocoumarin, nitrogen, and water content among six wild parsnip plants, however, accounted for only 46% of the variation in growth of webworms (Berenbaum et al., 1989), leaving open the possibility that other primary and secondary metabolites are important to survival and growth.

In this study, we examined the extent to which primary metabolites are involved in resistance of wild parsnip to its most important herbivore, the parsnip webworm. We set out to: (1) Quantify phenotypic variation in primary and secondary metabolites in parsnip buds, flowers, and fruits and determine the proportion of that variation that is attributable to additive genetic variation. We estimated narrow-sense heritabilities of both primary metabolites (nutrients) and secondary metabolites (allelochemicals) in order to determine the relative availability of these traits for herbivore selection. (2) Estimate the ability of webworms to reduce reproductive success and thus act as selective agents. For herbivores to act as selective agents on any plant trait, they must cause a reduction in plant fitness, either by reducing seed production or by causing outright mortality. To ascertain whether webworms can affect parsnip fitness, we compared seed production in the

presence and absence of webworms in which webworms were selectively eliminated by insecticide treatment. (3) Estimate the covariance between plant fitness and primary metabolites in parsnip buds, flowers, and fruits, both in the presence and absence of webworms. For primary metabolites to play a role in defense of parsnips against webworms, the covariance structure between plant fitness and primary metabolites should differ between plants that are free from webworms (sprayed with insecticide) and plants that are infested (not sprayed). Such analyses can identify the degree to which primary metabolites may be subject to selection by parsnip webworms.

METHODS AND MATERIALS

All experiments were conducted at the University of Illinois Phillips Tract Research area, located 3 km NE of Urbana, IL, where wild parsnips occur in large numbers throughout approximately 6 ha of old fields. The common garden plot employed for estimation of heritabilities was also located at Phillips Tract, but in an area that has been tilled every other year for the last 15 years. At this location, wild parsnips transition from a vegetative rosette to reproductive state during the first half of May. Parsnip webworm adults oviposit on the rosettes in late April and early May. The plants subsequently bolt, and the largest inflorescence, the primary umbel, unfurls at the apex of the main shoot, followed by the secondary umbels that unfurl at the tips of branches, and, later still, by the tertiary umbels that appear at the tips of secondary branches. Seeds ripen in mid- to late July.

Chemical Analyses. The secondary metabolites assayed were furanocoumarins (imperatorin, bergapten, xanthotoxin, sphondin, psoralen, and isopimpinellin), the coumarin osthol, terpenes (*cis*- and *trans*-ocimene, caryophyllene, bergamotene, cubebene, and farnesene), the phenylpropanoid myristicin, and three fatty acid derivatives (palmitolactone, octyl acetate, and octyl butyrate). The primary metabolites assayed were soluble protein, sugars (fructose, glucose, sorbitol, myo-inositol, and phytic acid), and saturated and unsaturated C_{18} fatty acids. With the exception of the sugars and phytic acid, we performed all analyses according to Zangerl et al. (1997)

Samples of buds and developing fruits that had been stored at -80°C were lyophilized and ground to a fine powder using a Wigglebug (Crescent Dental Manufacturing Co., Chicago, IL). Powdered aliquots were weighed (with amounts varying between 10 and 20 mg) and analyzed for content of secondary and primary metabolites. Separate aliquots were extracted for furanocoumarins, sugars and protein, and fatty acids and terpenes.

For the determination of soluble protein and sugar content, we extracted plant material with 300 μl of distilled water containing fucose as internal standard (1 mg/ml) inside a 1.5-ml centrifuge tube at room temperature for 1 hr. We then vortexed and centrifuged the samples at 12,200g for 10 min. Five microliters of

the supernatant were analyzed for protein, and two separate aliquots of 50 μl were transferred to 0.5-ml glass tubes for use in determination of sugar and phytic acid content. The spectrophotometric protein assay was performed according to Zangerl et al. (1997).

To one of the 50-μl sugar extracts, we added 1.5 units of phytase, which converts phytic acid to myo-inositol. Both sugar aliquots were incubated for 1 hr at 37°C. Samples were then dried under vacuum. We prepared the sugars for separation and quantification by gas chromatography (GC) by first derivatizing them. We added 300 μl of TriSil Z (Pierce) to each tube, sealed the tube inside a crimp top GC vial, and incubated the mixture at 70°C for 10 min. One microliter of each sample was analyzed by GC as described in Zangerl et al. (1997). Fructose, glucose, sorbitol, and myo-inositol were detected and quantified in all samples. The internal fucose standard was used to correct for variations in injection volume. The difference in myo-inositol between the phytase-treated and untreated samples was converted to phytic acid equivalents based on phytase-treated phytic acid standards.

Heritability Estimation. In May 1998 in a 1.3-ha field, all bolting wild parsnips that had primary umbels at the bud stage were marked with vinyl flagging tape. These plants served as maternal parents in controlled crosses. Later, when they were anticipated to have receptive female flowers within 3 days, we enclosed the primary umbel in a nylon bag of fine mesh (4 threads/mm) to prevent visitation by insect pollinators. As well, we marked all plants within the same field that had primary umbels at the bud stage for use as pollen donors for controlled crosses with the maternal plants. As each maternal plant became receptive, evidenced by the spreading of the two stigmatic surfaces, pollen was collected from a plant that was shedding pollen. Pollen was collected by lightly tapping the anthers onto a plastic Petri plate. Subsequently, it was transferred to the tip of a finger and applied to the stigmas of a receptive plant. We pollinated all of the flowers in 3–5 umbellets within the primary umbel of each maternal plant with pollen from a single donor, and then we marked each pollinated umbellet with a permanent black marker. The entire umbel was then enclosed again in a mesh bag. Forty pollinations were performed involving 80 plants. Of the 40 pollinations, 23 resulted in sufficient numbers of seeds for further study and were not contaminated by other pollen sources, that is, the pollinated umbellets yielded seeds, while those that were not hand-pollinated failed to set seed.

The seeds from these crosses were collected in late July 1998, soaked in water for 1 hr and kept in the cold room to facilitate germination before sowing in 1-l plastic pots filled with 1:1:1 peat:perlite:Drummer soil. The offspring were grown in the greenhouse at 24°C under 16 hr days (natural light was supplemented with 250-W metal halide lamps) until March 1999 when they were transplanted into the soil in 5 long rows with 0.5 m between plants and 1 m between rows. Transplanting early in the season allowed the plants to receive the chilling temperatures and short

daylengths required for flowering. In early May 1999, all plants were sprayed with the systemic insecticide acephate (Orthene, Ortho, Columbus, OH) to eliminate webworms. As primary umbel buds and half-filled fruits, fruits in which the endosperm spans half of the length of the fruit, became available (from mid-May to mid-June), they were sampled and placed inside 1.5-ml plastic centrifuge tubes, which were immediately submerged in liquid nitrogen and later taken to the laboratory and stored at −80°C.

Samples were prepared and analyzed as described earlier except that ethyl acetate was used to extract nonpolar constituents, including furanocoumarins, and analyzed by GC. The separation and quantification of furanocoumarins by GC was not as satisfactory as with HPLC; thus, heritability estimates were produced for only three furanocoumarins—xanthotoxin, psoralen, and bergapten. Additionally, quantification of phytic acid was omitted, as this constituent was highly variable due to the method of analysis and was unlikely to yield meaningful estimates of heritability.

We calculated heritabilities obtained from the full-sib families based on variance components extracted from ANOVA according to Becker (1984). Because family size varied, we obtained the between-family component of variance by using an adjusted family size k:

$$k = \left(N - \left(\sum n_i^2\right)/N\right)/(f - 1)$$

where N is total number of individuals, n_i is the number of siblings in the ith family, and f is the number of families (Becker, 1984).

Field Experiment; Webworm Impact on Fitness and Identification of Resistance Factors.

The field experiment consisted of comparing chemistry and fitness of wild parsnips in plots protected from webworms by spraying with plots experiencing natural infestations. Six square plots, each measuring 15 m on a side, were laid out in a field measuring 100 by 130 m. Parsnip plants in three randomly selected plots were sprayed with acephate in early May 1997. Because parsnip webworm adults lay their eggs in late April and early May, the timing of this insecticide application, which is effective for 2–3 wk, effectively eliminated all larvae for the duration of the season. Later in May, we marked all 317 plants in the six plots that had primary umbels at the bud stage. At that time, we tied a numbered vinyl flag to each of these plants, and a sample of buds was removed and placed inside a 1.5-ml centrifuge tube, which was submerged in liquid nitrogen and later stored in the laboratory at −80°C. We monitored marked plants every third day for the presence of half-filled fruits, which we sampled, froze, and stored in the same way as the buds.

As the fruits began to ripen, we scored the level of webworm infestation by counting the number of umbels of each order (1°, 2°, and 3°) exhibiting webworm feeding damage and assessing by visual inspection whether the damage was light (less than 25% of the umbel mass damaged), moderate (between 25% and 50% damaged), or heavy (greater than 50% damaged). In addition to numbers of infested umbels, we recorded the total numbers of umbels of each order. Plants were also scored for the presence of a disease, the symptoms of which included wilting and eventual necrosis of the umbels or stems supporting the umbels. The cause of the disease was identified by the University of Illinois Plant Clinic as a species of *Phomopsis*, possibly *Phomopsis diaehenii*. Plants that exhibited symptoms of this disease were dropped from further study, leaving 83 protected plants and 88 unprotected plants.

Because the order of seed ripening follows the order of flowering, ripe seeds are produced over a period of several days. Ripe seed from each plant was collected every third day and placed in a 237-ml waxed cardboard cup with tight-fitting lid. The cup was kept at the base of the plant until all of the seed was collected. At that time (late July–early August), the remaining dead and largely dry shoot portion was harvested, air-dried in the laboratory for 2 mo, and weighed. Seeds containing embryo and endosperm were separated from empty seeds, pieces of rays, and other small debris by winnowing. The seed from a single plant was released at the top of a 6.1-m long PVC pipe measuring 8.8 cm in diam. The opening at the bottom of the pipe was situated 5 cm above the floor. As the seed material began to emerge from the bottom of the pipe, a sheet of continuous feed computer paper was steadily dragged along the floor under the pipe. Viable seeds, those containing endosperm, invariably emerged first, followed by empty seeds and debris. The distribution of each batch of seed along the paper was visually inspected, and the dividing line between seeds containing embryo and endosperm and nonviable seeds and debris was identified. All of the viable seed was then collected and weighed to estimate reproductive success.

Statistical Analyses of Field Experiment. To determine whether webworm infestation affected parsnip fitness, we regressed mass of seeds against vegetative shoot mass separately for sprayed and unsprayed plants. Analysis of covariance with seed mass as the dependent variable and shoot mass as the covariate was employed to determine whether the slopes of the regressions differed from one another, as would be evidenced by a significant covariate by treatment interaction. Because webworms feed only on reproductive parts, they can be expected to affect reproductive effort. To establish that webworms account in large part for differences in reproductive success between sprayed and unsprayed plants, we regressed realized reproductive effort (mass of seeds divided by vegetative shoot mass) against an index of webworm damage (the severity of damage times the proportion of umbels damaged).

To determine whether plant fitness was affected by differential production of chemical constituents, we performed two separate analyses. First, we determined which chemical constituents were correlated with fitness, as measured by reproductive effort, separately for protected and unprotected plants. The second analysis was employed to examine the effect of configurations of different traits that would not be detected by linear regression due to epistatic interactions. The approach was first to identify common "chemotypes" by use of cluster analysis (K-means cluster analysis procedure in SPSS). To simplify the analysis and take into account positive correlations within a chemical class, we summed the constituents within classes to yield measures of total sugar and total fatty acid. Similarly, because the linear furanocoumarins were correlated with one another, a summed total was used. Correlations among the other constituents were not significant, and so these were not grouped. All variables were first standardized by z-transformation. We performed several iterations starting with two clusters and incrementing by one up to seven clusters. The iteration that yielded the greatest number of clusters with sufficient representation of both protected and unprotected plants to allow statistical comparisons between treatments was chosen for further investigation. Within each of the suitably populated clusters, we compared reproductive effort between sprayed and untreated plants. Clusters in which reproductive effort was not affected by insecticide spray were judged to be resistant chemotypes, while those in which the unsprayed plants had lower reproductive effort than treated plants were judged to be susceptible chemotypes. Comparisons were made among clusters to determine which chemicals differed significantly and thus might contribute to resistance or susceptibility.

RESULTS

Heritabilities. Significant heritabilities, as judged by significant family effects in the analysis of variance, were observed for unsaturated C_{18} fatty acids in both buds and developing fruits, stearic acid in developing fruits, fructose, and sorbitol in buds, fructose in fruits, myo-inositol in fruits, and bergapten and psoralen in fruits (Table 1). Similar levels of genetic variation in bergapten content were previously detected in wild parsnip seeds (Berenbaum et al., 1986; Zangerl and Berenbaum, 1990).

Field Experiment—Webworm Impact on Fitness and Identification of Resistance Factors. Chemical composition of parsnip buds and half-filled fruits was largely insensitive to spray treatment. With three exceptions, spray treatment had no significant effect on chemical composition of buds and developing fruits. In unsprayed plants, there were lower concentrations of isopimpinellin in buds (0.155 vs. 0.218 μg/mg, treatment $P = 0.001$ from ANOVA with plot nested in treatment) and developing fruits (0.694 vs. 1.100 μg/mg, treatment $P = 0.005$ from ANOVA with plot nested in treatment) and a higher level of soluble fruit protein

TABLE 1. FULL-SIB HERITABILITIES OF PRIMARY AND SECONDARY METABOLITES IN WILD PARSNIP BUDS AND FRUITS

Constituent	Bud (h^2)	Fruit (h^2)
Primary metabolites		
Glucose	0.144	0.042
Fructose	0.342*	0.428*
Sorbitol	0.306*	0.116
Myo-inositol	0.048	0.282*
Unsaturated C_{18} fatty acids	0.382*	0.226*
Stearic acid	0.429*	0.000
Secondary metabolites		
Bergapten	0.138	0.426*
Xanthotoxin	−0.048	0.160
Psoralen	0.046	0.728*
Myristicin	0.234	n.p
Palmitolactone	0.158	−0.038
Osthol	n.p.	0.152

Note. Total number of individuals in the analyses ranged from 119 to 126 (variation due to sample losses). n.p. constituent not present.
*Significant family effect in ANOVA, $P < 0.05$.

(79.53 vs. 63.6 μg/mg, treatment $P = 0.038$ from ANOVA with plot nested in treatment). Similarly, there were only three constituents for which a significant plot effect was detected—bergapten and total furanocoumarins in developing fruits and bergamotene in buds (all Ps < 0.05, data not shown).

In terms of reproductive effort (seed mass/shoot vegetative mass), sprayed plants converted 2.5 times more biomass to seed than plants that were not sprayed (Figure 1, significant interaction between shoot mass and treatment in an analysis of with seed mass as the dependent variable and shoot mass as the covariate, $F = 47.17$, $P < 0.001$). That spray treatment might affect vegetative mass and, therefore, render reproductive effort difficult to interpret was not the case, as spray treatment had no effect on shoot biomass ($P = 0.927$) in an ANOVA with plot nested in treatment. That webworm damage largely accounts for the variation in reproductive effort is evidenced by a significant inverse relationship between reproductive effort and the index of webworm damage (Figure 2) and by an average damage index value among the unsprayed plants that accounts for a decline in reproductive effort from mean of the sprayed plants of about 50%, comparing favorably with the 61% reduction in seed production in Figure 1.

As to whether plant fitness is affected by differential production of primary or secondary metabolites, for all but four of the plant constituents there was no relationship between concentration in buds or half-filled fruits and the ability of plants to convert biomass to viable seed (reproductive effort—seed mass/shoot mass). The exceptions were limited to constituents of half-filled fruit—osthol,

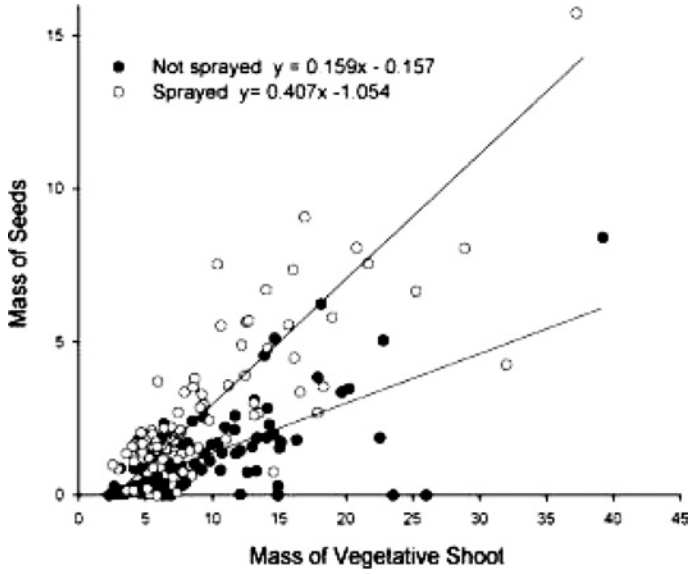

FIG. 1. Regressions of viable seed mass against shoot mass for wild parsnips sprayed with insecticide ($df = 1, 87$; $P < 0.001$; $r^2 = 0.656$) and for unsprayed parsnips ($df = 1, 81$; $P < 0.001$; $r^2 = 0.416$).

sorbitol, protein, and total furanocoumarins (Table 2). Reproductive effort was negatively associated with osthol in sprayed plants, suggesting a metabolic cost for its production; no such association was found with osthol in unsprayed plants, indicating that whatever costs are associated with its production were compensated for by its benefit in the presence of webworms. The same pattern was observed for fruit protein, suggesting that excess allocation of resources to protein may be costly in sprayed plants, but costs to such excess allocation are compensated for in unsprayed plants. Sorbitol appears to enhance susceptibility, as it has a positive relationship with reproductive effort in the sprayed plants and no relationship in unsprayed plants. Lastly, furanocoumarins appeared to exact no cost in sprayed plants but were positively associated with fitness in unsprayed plants.

A cluster analysis, generating six clusters based on plant chemical characteristics, yielded four clusters containing 98% of the 105 individuals with complete chemical profiles. These clusters contained sufficient numbers of individuals (between 12 and 53) to allow comparisons of fitness between sprayed and unsprayed plants. Two of these clusters, or chemotypes, numbered 1 and 3, were identified as being susceptible because unsprayed plants had significantly lower reproductive effort than sprayed plants (Figure 3). The two remaining chemotypes were judged to be resistant, because we found no significant differences in reproductive

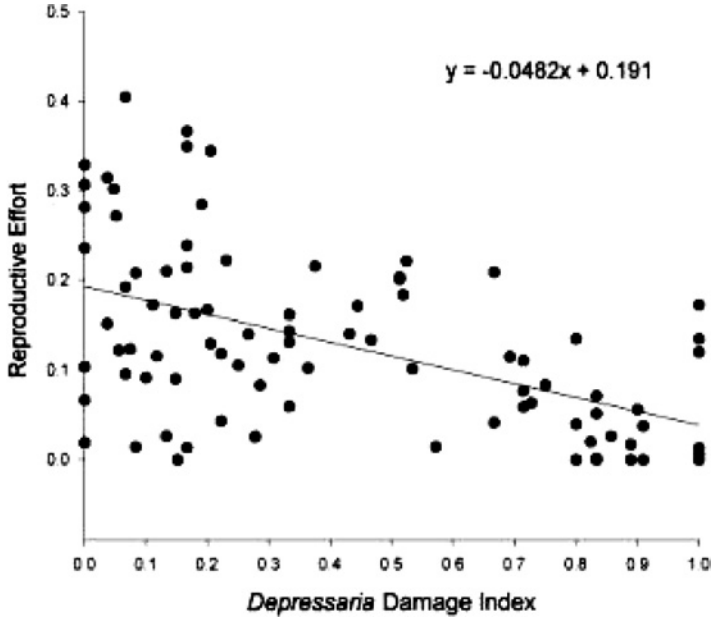

FIG. 2. Regression of reproductive effort (total seed mass/vegetative shoot mass) as a function of parsnip webworm damage, where damage is an index calculated as the severity of damage (with values ranging from 0 to 3) times the proportion of umbels damaged ($df = 1, 81$; $P < 0.001$; $r^2 = 0.214$).

effort between sprayed and unsprayed plants. The chemotypes differed significantly for 14 of the 22 constituents, including four nutrients. Constituents that distinguished resistant individuals from susceptible ones were high levels of osthol, octyl butyrate, and fatty acids in developing fruit, as well as high levels

TABLE 2. REGRESSION COEFFICIENTS (% VARIATION EXPLAINED) FOR CHEMICAL CONSTITUENTS IN WILD PARSNIP THAT EXHIBITED SIGNIFICANT RELATIONSHIPS WITH REPRODUCTIVE EFFORT IN SPRAYED OR UNSPRAYED PLANTS

Constituent	Sprayed plants	Unsprayed plants
Fruit osthol	−0.180* (8.6)	0.012 (0.3)
Fruit protein	−1.55* (7.5)	0.0004 (0.1)
Fruit sorbitol	0.040* (7.5)	−0.023 (0.5)
Fruit furanocoumarins	0.00205 (0.4)	0.0054* (5.6)

* $P < 0.05$.

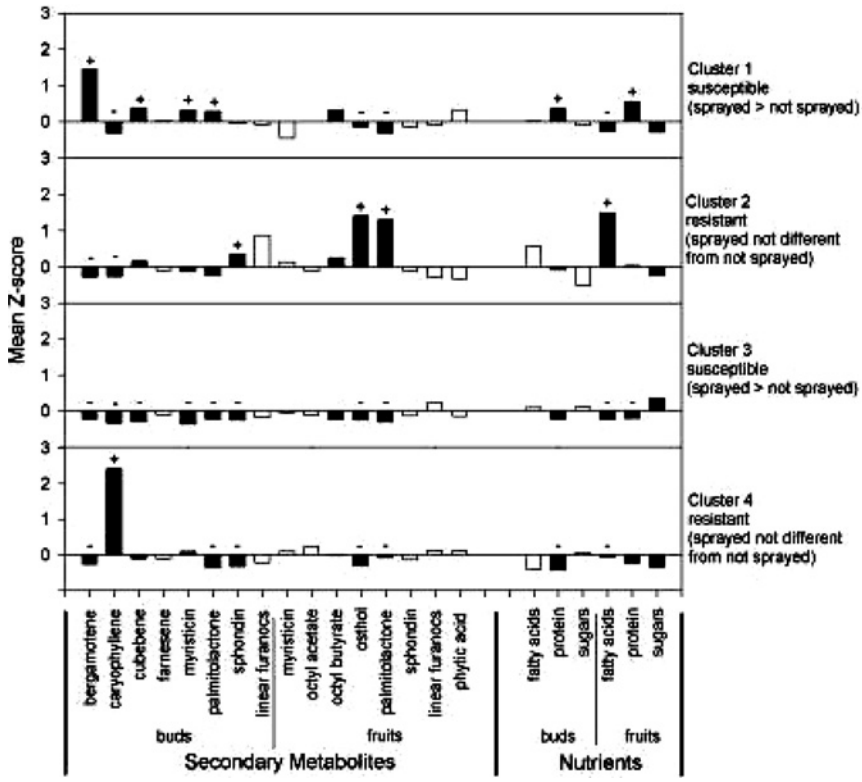

FIG. 3. Chemical phenotypes of wild parsnip identified through cluster analysis based on both primary and secondary metabolites. Blackened bars represent constituents that differ significantly among clusters. *Post hoc* Bonferroni-adjusted comparisons are represented by + and −. Plusses and minuses are significantly different from one another, but not different from unmarked bars.

of linear furanocoumarins in buds (cluster 2 vs. clusters 1 and 3) and high bud caryophyllene combined with low bud protein (cluster 4 vs. clusters 1 and 3). Conversely, susceptibility was associated with overall average chemical composition (cluster 3) and with high levels of bergamotene, cubebene, myristicin, and palmitolactone in buds and high protein in both buds and fruit (cluster 1 vs. others). We did not detect any costs of defense for resistant chemotypes, as evidenced by a lack of significant differences in reproductive effort among the four clusters for sprayed plants ($P = 0.427$ from one-way ANOVA).

The potential epistatic nature of these traits was underscored by a comparison of a separate set of clusters based solely on primary metabolites. Three of the six clusters contained 84% of the individuals having complete chemical profiles

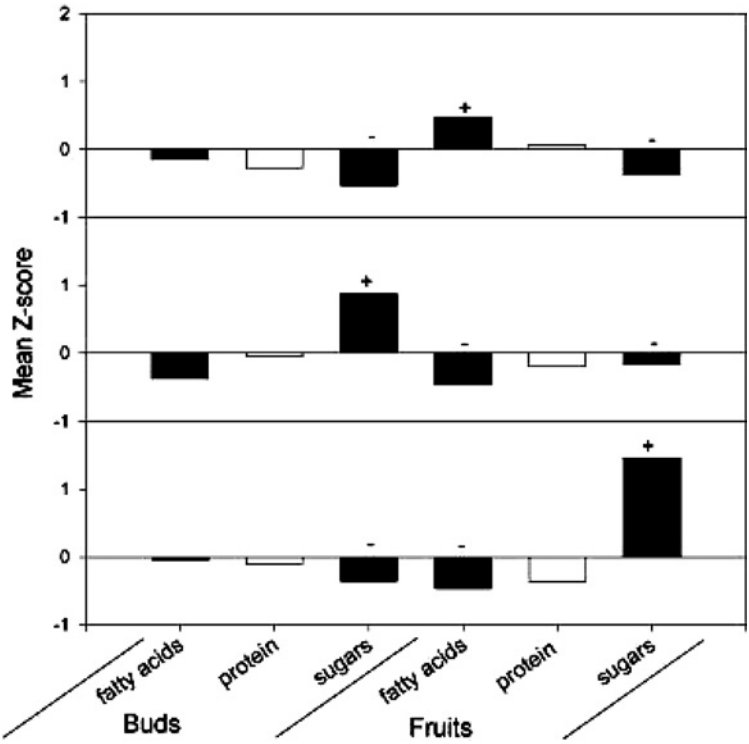

FIG. 4. Chemical phenotypes of wild parsnip identified through cluster analysis based only on primary metabolites. Black bars represent constituents that differ significantly among clusters. *Post hoc* Bonferroni-adjusted comparisons are represented by + and −. Plusses and minuses are significantly different from one another, but not different from unmarked bars. None of the three phenotypes were resistant to herbivores as within each phenotype reproductive effort was significantly reduced in plants that were not sprayed compared to plants that were sprayed (t-tests, all Ps < 0.05).

(119) as well as sufficient numbers of both sprayed and unsprayed individuals (between 20 and 50) for comparisons. Four of the six primary metabolite traits (protein, sugar, and fatty acid in buds and half-filled fruits) differed significantly among clusters; however, after Bonferroni adjustment, differences between pairs of clusters were detected for only three of the six traits (Figure 4). Despite differences in fruit sugar and fatty acid content and bud sugar content, there were no differences in resistance; within each cluster, unsprayed plants had reduced reproductive effort compared to sprayed plants (all Ps < 0.05). Thus, the variation in resistance associated with variations in primary and secondary metabolites in Figure 3 disappeared when only primary metabolites were considered.

DISCUSSION

Significant costs and benefits detected independently of other plant constituents were found only for four fruit constituents (Table 2). Consistent with previous studies (Berenbaum et al., 1986, 1989; Zangerl and Berenbaum, 1993), evidence of the role of furanocoumarins in resistance of wild parsnips to parsnip webworms was found in this study. Among parsnips that were not sprayed, those that successfully converted a higher proportion of biomass to seed production contained higher levels of half-filled fruit furanocoumarins (Table 2). Sprayed plants, by comparison, exhibited no relationship between reproductive effort and half-filled fruit furanocoumarin concentration, indicating an absence of costs at this stage of development. Costs of furanocoumarin production have, however, been detected in the final stage of ripe seeds (Zangerl and Berenbaum, 1997). Whether this absence of detectable costs of furanocoumarin production in half-filled fruits is real or merely difficult to detect is not clear. Ripe seeds are easily staged and do not subsequently change in concentration, whereas half-filled fruits, as measured in this study, mark a stage in development when furanocoumarin accumulation is rapid (Zangerl and Nitao, 1998); slight differences in fill rates among phenotypes could result in rather large variations in concentration. Consequently, the absence of costs of furanocoumarins in half-filled fruits, and the weak benefits recorded in this study, may simply reflect the imprecision of staging a dynamic point in development.

The other three chemical constituents in half-filled fruits that were linked to fitness independent of other chemical traits were osthol (a 7-hydroxycoumarin derivative), sorbitol (a sugar alcohol associated with susceptibility), and soluble protein, which was associated with resistance. Although osthol itself has not previously been linked specifically to insect resistance in plants, other hydroxycoumarins are known to have antifeedant and/or toxic effects on insects (Dreyer et al., 1987; Patton et al., 1997). That all four of the constituents influencing fitness in the presence of webworms are metabolites of half-filled fruits and not buds might be due to the fact that wild parsnips can more readily compensate for losses of buds than fruits (Nitao and Zangerl, 1987). Indeed, furanocoumarin levels in buds are far lower than in any subsequent developmental stage (Nitao and Zangerl, 1987).

Remarkably, there was only one significant correlation among total sugar, total furanocoumarin, total fatty acid, soluble protein, and total terpenoids in buds and fruits; that correlation was slight and was between bud sugar and bud furanocoumarin content ($r = -0.184$, $P = 0.026$, $N = 148$). Thus, chemical classes can vary independently of one another. Chemical traits whose effects on resistance depend on other chemical traits display epistasis. Such effects cannot be detected by standard regression techniques, but comparisons of resistant and susceptible phenotypes suggest interactions (Figures 3 and 4). In this study, phenotypes

differentiated from one another based solely on primary metabolites were all susceptible to webworms. These phenotypes were distinguished from one another by combining high sugar content with low (nutritive) fatty acid content or low sugar content with high fatty acid content (Figure 4). It appears that webworms thrive on plants with high energy content in one form or another.

When primary nutrients are considered together with secondary metabolites, the effects of sugar and fatty acids are less clear. The most distinctive characteristics of the resistant phenotype described by cluster 2 (Figure 3) are high fatty acid content combined with high levels of osthol and palmitolactone. Another resistant phenotype, described by cluster 4, is distinctive for high caryophyllene content combined with below-average amounts of several primary and secondary metabolites. Susceptible phenotypes were characterized either by moderately below-average amounts of several of the secondary metabolites and two primary metabolites (cluster 3) or a combination of generally above-average amounts of terpenes, moderately below-average amounts of osthol and palmitolactone, above-average bud fatty acids, and above-average fruit protein (cluster 1).

Collectively, results from this study suggest that, although there are primary metabolites that display genetic variation, selection by herbivory on primary metabolism likely occurs as a result of interactions between primary and secondary metabolites. This finding is consistent with previous reports of amelioration or exacerbation of toxicity of secondary metabolites in the presence of nutrients (Slansky, 1992). Primary metabolism of plants may be more constrained in its ability to respond to herbivore selection given the potentially conflicting selection pressures imposed by the physiological demands for nutrients in the plant. Notwithstanding, our study shows that plant resistance to insects can best be understood by considering the collective impact of plant tissue composition on herbivore behavior and physiology. Secondary metabolites exist within a matrix of primary metabolites, and it is this complex matrix that determines the selective impact of herbivory on plant chemistry.

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DIRECT EVIDENCE FOR MEMBRANE TRANSPORT OF HOST-PLANT-DERIVED PYRROLIZIDINE ALKALOID *N*-OXIDES IN TWO LEAF BEETLE GENERA

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Abstract—The chrysomelid leaf beetles *Longitarsus jacobaeae, Oreina cacaliae*, and *O. speciosissima* sequester pyrrolizidine alkaloids from their asteracean host plants and store them as nontoxic *N*-oxides. Previous analyses showed that *Longitarsus* is able to *N*-oxidize protoxic tertiary PAs, but did not resolve in which form *N*-oxides are taken up. For *Oreina*, beetles seem able to directly transmit the polar PA *N*−oxides from the gut into the hemolymph and prevent any reduction of them in the gut yielding protoxic free bases. Here, we confirm the predicted direct uptake of PAs as *N*−oxides by *Oreina*, and elucidate the situation for *Longitarsus* by applying double-labeled [^{14}C]senecionine [^{18}O]*N*−oxide as tracer. The beetles were fed with the tracer and subsequently senecionine *N*−oxide was recovered from the defensive secretions (*Oreina*) and beetle extracts (*Longitarsus*), purified by HPLC, and submitted to ESI-MS, GC-MS, and analysis of the specific radioactivity. The ^{18}O-label is retained without any loss in the labeled senecionine *N*−oxide recovered from the two *Oreina* species. Analysis of the *Longitarsus* experiment was complicated by a contamination of the HPLC-purified senecionine *N*−oxide with a second compound, identified as a dihydrosenecionine *N*−oxide by high-resolution CID analysis.The dihydrosenecionine *N*−oxide, probably the 15,20-dihydro derivative,

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constitutes a major idiosyncratic senecionine metabolite present in the beetle. The recovered senecionine $N-$oxide retained 74% ^{18}O-label. The remaining 25% is mostly due to loss of ^{18}O by reduction and subsequent re-$N-$oxidation. The experiments confirm for both beetle genera a direct uptake of the polar nontoxic PA $N-$oxides, which requires specific membrane carriers. Accumulation of detrimental free base PA is prevented by glucosylation (*Oreina*) or $N-$oxidation (*Longitarsus*).

Key Words—Sequestration, pyrrolizidine alkaloid, chemical defense, membrane transport, alkaloid *N*-oxide carrier, *Longitarsus*, *Oreina*, Chrysomelidae, Coleoptera.

INTRODUCTION

Pyrrolizidine alkaloids (PAs) are plant secondary metabolites that are potentially toxic and protect against herbivory. In plants, PAs are usually present as *N*-oxides (Figure 1B), a form that is hydrophilic and *a priori* nontoxic. When ingested by a herbivore, PA *N*-oxides are generally reduced to lipophilic tertiary alkaloids (free bases; Figure 1A) and passively absorbed into the herbivore's body (Hartmann, 1999). In vertebrates, these tertiary PAs are converted into pyrrolic metabolites that easily react with biological nucleophiles causing hepatotoxic and pneumotoxic effects (Mattocks, 1986; Cheeke, 1989; Winter and Segall, 1989). In insects, severe detrimental effects have similarly been described, ranging from mutagenesis, i.e., *Drosophila* (Frei et al., 1992), to lethal developmental failures in nonspecialist phytophagous insects like *Philosamia ricini* (Narberhaus et al., unpublished). However, a number of specialized insects from diverse taxonomic groups have developed adaptations that allow sequestration of PAs from plants, and their accumulation and utilization for antipredator defense (Hartmann, 1999; Hartmann and Ober, 2000). Although the hurdle that PAs' toxicity imposes on insects is the same, the strategies evolved to overcome it differ in various taxa. Larvae of the arctiid moths *Creatonotos transiens* and *Tyria jacobaeae* and the grasshopper

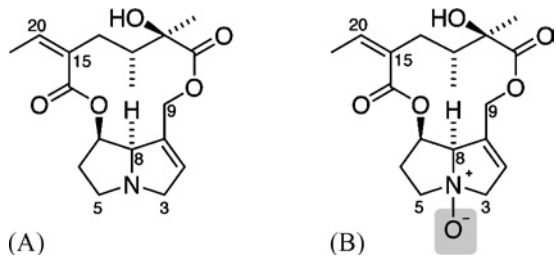

FIG. 1. Structures of the two forms of senecionine: (A) tertiary alkaloid (free base), (B) its *N*-oxide; in the [^{14}C]senecionine [^{18}O]*N*-oxide applied as double-labeled substrate the position of ^{18}O is shaded, the carbons 3, 5, 8, and 9 are ^{14}C-labeled.

Zonocerus variegatus passively take up reduced PAs and detoxify them by *N*-oxidation. In *T. jacobaeae*, a specific flavin-dependent monooxygenase (FMO) in the hemolymph is responsible for this reaction (Lindigkeit et al., 1997; Naumann et al., 2002). Some vertebrates, like guinea pigs (Cheeke, 1994) and sheep (Huan et al., 1998), are also known to *N*-oxidize tertiary PAs through vertebrate-specific multisubstrate FMOs, making these highly polyphagous grazers resistant to the toxicity of PA food-plants.

A variety of different sequestration strategies is found in the beetle family Chrysomelidae. The neotropical Chrysomelinae species *Platyphora boucardi*, for example, takes up tertiary PAs as they are supplied by its host plants (Pasteels et al., 2001; Pasteels et al., 2003). They are absorbed and transported into defensive glands where they are stored unchanged as tertiary alkaloids. The efficient transport prevents accumulation of detrimental concentrations in the hemolymph and body tissues (Hartmann et al., 2001). The adapted alticine leaf beetles of the genus *Longitarsus N*-oxidize tertiary PAs and store them mainly in their *N*-oxide form (Narberhaus et al., 2003), similar to the way the arctiid moths handle the alkaloids. Observations also indicate the existence of a FMO located in the beetles' hemolymph (Narberhaus et al., unpublished data), analogous to the one responsible for *N*-oxidation in *T. jacobaeae*. In contrast to the arctiid moths, *Longitarsus* also stores minor amounts of PAs in the tertiary form. This portion appears to be separated from the enzyme in unidentified storage tissues and remain there unchanged for prolonged time periods (Narberhaus et al., 2004). In arctiids, only tertiary PAs permeate the gut membrane, but the situation in *Longitarsus* is unclear. The possibilities would be either a reduction in the gut followed by passive uptake of tertiary PAs across the gut membrane and reoxidation, or, as demonstrated for leaf beetles of the alpine genus *Oreina*, a suppression of reduction in the gut combined with an active transport of *N*-oxides into the body. *Oreina* leaf beetles also store PAs in the *N*-oxide form and utilize them in their defensive secretions. Since *N*-oxidation has not been detected in these beetles, it seems probable that *Oreina* possesses both a carrier in the gut membrane that transports the hydrophilic *N*-oxides unchanged into the hemolymph, and a carrier that transports them from the body into the defensive glands (Hartmann et al., 1999). Even though biochemical evidence is strong, no direct proof for the state in which PAs cross membranes in *Oreina* has hitherto been brought forth.

The goal of the current investigation was to elucidate the process of membrane transfer of PAs in *Longitarsus* and *Oreina*. To establish the molecular state in which PAs cross membranes, only one experiment can provide direct evidence, i.e., the oral administration of a PA *N*-oxide with a labeled *N*−oxide oxygen atom. We, therefore, prepared [^{14}C]senecionine [^{18}O]*N*-oxide (Figure 1B) from tertiary [^{14}C]senecionine, using a senecionine-*N*-oxygenase obtained from the hemolymph of *T. jacobaeae* caterpillars. As exemplary PA-sequestering species, we fed *Longitarsus jacobaeae*, *Oreina speciosissima*, and *O. cacaliae*

with this tracer. All three species feed on PA containing plants. *L. jacobaeae* is specialized on the asteracean genus *Senecio* and has already been shown to efficiently *N*-oxidize PAs in tracer feeding and injection experiments (Narberhaus et al., 2003). This beetle species can be regarded as a highly adapted PA plant feeder, which is corroborated by the PA specificity of the *N*-oxygenase. The two *Oreina* species have hosts in the asteracean genera *Petasites, Senecio*, and *Adenostyles*, and have been well-investigated for their PA sequestration mechanisms. Here, they were further analyzed to complete our knowledge on PA uptake mechanisms in these species and to serve as comparison to *Longitarsus*.

METHODS AND MATERIALS

Biosynthesis of [^{14}C]Senecionine [^{18}O] N-*Oxide*

[^{14}C]Senecionine (1.07–1.34 GBq·mmol^{-1}) was prepared biosynthetically from [1,4-^{14}C]putrescine-dihydrochloride (4.1 GBq·mmol^{-1}; Amersham Biosciences, Freiburg, Germany) using root cultures of *Senecio vulgaris* according to Hartmann (1994). The preparation of [^{14}C]senecionine [^{18}O]*N*-oxide was performed according to a slightly modified protocol of Lindigkeit et al. (1997). Senecionine *N*-oxygenase from hemolymph of *T. jacobaeae* was used to catalyze the *N*-oxidation. To isolate the enzyme, late instar caterpillars of this species were caught in the Meijendel dunes (Leiden, NL) from their hosts in June 2002. About 280 larvae were used to collect 8.4 ml of hemolymph which was precipitated in a slowly stirred saturated ammonium sulfate solution at 4°C. After centrifugation, the supernatant was discarded, and the remaining protein pellet was stored at −80°C without losing its activity. Before use, the enzyme was desalted *via* Sephadex G-25 (PD-10 columns, Amersham Biosciences, Freiburg, Germany). A gas-tight glass apparatus was used, consisting of a 100-ml round-bottomed flask that was connected to an $^{18}O_2$ gas flask (99% purity, 95% ^{18}O excess) on one side and to a vacuum pump on the other side. Approximately 9-mg senecionine (Roth, Karlsruhe; 100% pure according to GC) were suspended in 5-ml 0.01 M phosphate buffer, pH 2; after dissolving, the solution was adjusted to pH 7.0 with 0.01 M K_2HPO_4 containing 2 mM dithioerythritol. The final volume was adjusted to 25 ml, and 30-μl [^{14}C]senecionine (10^7 cpm) in methanol was added. Finally, the desalted enzyme solution containing crude senecionine *N*−oxygenase in 0.01 M potassium phosphate buffer (pH 7.0) was added. All buffers and solutions had been thoroughly degassed with helium before use. The top was sealed with a Teflon septum and flushed and evacuated ×10 with > 99.9% N_2. After one flush with $^{18}O_2$ followed by evacuation, the round flask was filled with $^{18}O_2$, and the reaction was started by injecting 1-ml degassed reaction buffer containing 50-mg NADPH through the septum. The apparatus was shaken in a 30°C water bath for

24 hr before the reaction was stopped by injection of 3-ml 25% HCl. Precipitating protein was removed by centrifugation. A small aliquot of the supernatant was subjected to TLC and analyzed using a multichannel radioactivity detector (Rita-32a, Raytest). The enzymatic *N*−oxidation turned out to be quantitative; the applied substrate senecionine was no longer detectable in the assay. The remaining solution was dried under an air stream, redissolved in 6-ml $CHCl_3$, and applied to a 9-g Al_2O_3 column. After flushing the column several times with a total of 20-ml $CHCl_3$, alkaloid *N*-oxides were eluted with the solvent MeOH:$CHCl_3$ (3:7 by volume). A total of $3 \cdot 10^6$ cpm (about 5.5 mg) was recovered. The ^{18}O-content of the sample, as well as of the beetle extracts, was determined by mass spectrometry after the feeding trial. Specific radioactivity was measured by scintillation counting and quantitative GC of a Zn/H^+-reduced aliquot. Using heliotrine as an internal standard, the yield was measured as $1.94 \cdot 10^8$ cpm/mmol (equal to $5.8 \cdot 10^5$ cpm/mg [^{14}C]senecionine or $5.5 \cdot 10^5$ cpm/mg [^{14}C]senecionine [^{18}O] *N*-oxide).

Tracer Feeding Experiments. Beetles of three species were caught in the field. *L. jacobaeae* was collected in St. Imier (Ct. Jura, CH) on *Senecio jacobaea* in August 2002, *O. cacaliae* on the Stöckalp (Ct. Obwalden, CH) on *Adenostyles alliariae* in July 2002, and *O. speciosissima* on the Gfelalp (Ct. Bern, CH) on *A. alliariae* and *Petasites albus* in August 2003. All insects were kept under long-day conditions at 18°C in plastic cylinders on their host leaves until use. To reduce the risk of complications with background PAs in the insects, the individuals of both *Oreina* species were fed with *P. albus*, whose leaves are practically free of alkaloids, for 2 wk before start of the feeding experiments. For the tracer feeding, a methanolic solution of [^{14}C]senecionine [^{18}O]*N*-oxide was painted on leaves of *S. jacobaeae* (for *L. jacobaeae*) or *P. albus* (for *O. cacaliae* and *O. speciosissima*). The subsequent procedures differed between species, depending on how well the insects ingested the leaves and on whether secretions were collected. With *L. jacobaeae*, four replicate experiments were performed with groups of 50 beetles each that were placed together in Petri dishes (day 0). Each group fed on leaf disks covered with 0.25 mg ($1.4 \cdot 10^5$ cpm) of the tracer. On day 2, the beetles had consumed the treated leaves completely and were placed on nontreated fresh leaves in clean containers. This step was repeated on day 4. On day 7, the beetles were killed by freezing and stored at −20°C until extraction, since no secretions could be collected.

Similarly, four replicate experiments were initiated with *O. cacaliae*. Each consisted of a group of three individuals that were placed into a Petri dish (diam 9 cm) and fed with a 3 × 3 cm *P. albus* leaf covered with 0.25-mg ($1.4 \cdot 10^5$cpm) [^{14}C]senecionine-[^{18}O]*N*-oxide. Beetles were transferred to new Petri dishes with untreated leaves on days 3 and 5. After 1 wk defensive secretions were collected by disturbing the animals with a pair of forceps and by wiping exuding liquid from

elytra and pronota with a piece of filter paper. Secretions were stored in methanol in a centrifuge tube at −20°C.

Seven individuals of *O. speciosissima* were collected. They were placed together into a Petri dish and fed with 1-mg ($5.5 \cdot 10^5$ cpm) [^{14}C]senecionine [^{18}O]*N*-oxide painted on a 5 × 5 cm leaf piece of *P. albus*. All was consumed on day 3. On days 3 and 5, beetles were transferred to a new Petri dish with a fresh untreated leaf. On day 8, the defensive secretions were collected from the insects.

Extraction of L. jacobaeae *Beetles.* Frozen beetles were ground in a mortar and extracted with 2-ml MeOH. After centrifugation, the supernatant was recovered, and the residual pellet was extracted twice with 2-ml MeOH. The combined solutions were dried under an air stream, and the residue was dissolved in 2-ml MeOH. Small aliquots of these crude extracts were subjected to scintillation counting. A total radioactivity of $2.48 \cdot 10^5$ cpm was recovered. Extracts were further purified through Al_2O_3 columns. After drying and redissolving the residue in 500-μl CH_2Cl_2, the extracts of the four beetle groups were each applied on 300-mg Al_2O_3. To remove tertiary PAs, the columns were washed with 30-ml CH_2Cl_2 before *N*-oxides were successively eluted with four 500-μl portions CH_2Cl_2:MeOH (7:3, by volume). The resulting fractions 1–4 were collected separately. A TLC analysis revealed the necessity of an additional washing to remove residual tertiary PAs. Therefore, fractions 1 and 2, which contained most of the radioactivity, were pooled, dried, and dissolved in 40-μl H_2O. Remaining tertiary PAs were removed by extraction with 40-μl toluene. The aqueous phase containing the PA *N*-oxide was recovered, dried, and subjected to HPLC for separation of senecionine *N*-oxide from other contaminant PAs, such as seneciphylline *N*-oxide. HPLC was performed using an RP-18 column (Nucleosil 120-5 C18, 250 mm long, 4-mm i.d., Macherey & Nagel). Samples were redissolved in 20-μl MeOH and injected into a 20-μl loop. Separation was achieved using helium-washed acetonitrile and H_2O/CF_3COOH (pH2) as solvents (2:8, by volume) at a flow rate of 1 ml/min, and UV detection was by absorbance at 210 nm. The retention time (RT) for senecionine *N*-oxide is 9.2 min. Fractions with the highest radioactivity (RT 8–10 min) were pooled separately for each of the four replicates. They contained on average a radioactivity of 20,600 cpm, corresponding to 37.9-μg labeled senecionine *N*-oxide. The solvent was removed by evaporation, and the samples were subjected to MS analysis.

Purification of Defensive Secretions from O. cacaliae *and* O. speciosissima. Filter papers with secretions were extracted ×3 each with 500-μl MeOH, evaporated, and redissolved in 200-μl MeOH. Total radioactivity recovered for the pooled extracts of the secretions of *O. cacaliae* was $1.8 \cdot 10^4$ cpm, while that of *O. speciosissima* secretions was $1.1 \cdot 10^4$ cpm. Purification of senecionine *N*-oxide was performed *via* HPLC following the procedure described above for

L. jacobaeae. Fractions of retention time 9–10 min, containing 4400 cpm (equivalent to 7.9-μg labeled senecionine *N*-oxide) in the *O. cacaliae* sample and 6500 cpm (equivalent to 11.7-μg labeled senecionine *N*-oxide) in the *O. speciosissima* sample, were evaporated and subjected to MS analysis.

Determination of ^{18}O Abundance in Senecionine N-*Oxide by Electrospray Mass Spectrometry.* Mass spectrometric analysis was carried out on a MAT 95 XL Trap hybrid tandem mass spectrometer (Thermofinnigan MAT, Bremen, Germany) equipped with the standard electrospray interface supplied by the manufacturer. The instrument consists of a double focussing sectorfield mass spectrometer with high-resolution and accurate mass measurement capabilities, coupled to a quadrupole ion trap mass analyzer. The second mass analyzer was used to investigate the fragmentation behavior of monoisotopically isolated precursor ions from the first mass analyzer by collision-induced dissociation (CID).

A reference sample of senecionine *N*-oxide, a reference sample of ^{18}O-labeled senecionine *N*-oxide, and the dry samples obtained by radio-HPLC were reconstituted with 100 μl of HPLC-grade acetonitrile, and directly analyzed by ESI MS. For all low-resolution measurements, a slightly modified version of the microspray device supplied with the instrument was used for sample introduction[1] and operated at a flow rate of approximately 0.8 μl/min and a spray voltage of 1.5–1.7 kV. The temperature of the heated capillary was set to 195°C. The remaining voltages of the electrospray interface were optimized for maximum signal intensity of the protonated molecular ion of senecionine *N*-oxide at $m/z = 352$. The mass spectrometer was operated at a resolution of 3000 (10% valley definition) and magnetically scanned from 50 to 1200 amu at a scan speed of 2 sec/dec. About 50 spectra for each sample were acquired in centroid mode and subsequently averaged.

For high-resolution and accurate mass measurements, samples were introduced by a nanospray device using gold-coated tapered nanospray emitters with approximately 2-μm i.d. tip openings (MasCom, Bremen, Germany) operating at a flow rate of approximately 50 nl/min and a spray voltage of 1.0–1.1 kV. The temperature of the heated capillary was set to 120°C. The remaining voltages of the electrospray interface were optimized as described above.

For accurate mass measurements employing the peak match method, the resolution was adjusted to 7000 (10% valley definition). An appropriate amount of a polypropylene glycol standard solution serving as an internal mass calibrant

[1]The original fused silica sample transfer capillary was replaced by stainless steel tubing of similar dimensions (SMS Service für Massenspektrometrie GmbH, Idstein, Germany). The nonconductive ferrule that holds the capillary was replaced by a graphite ferrule of appropriate size.

was added and mixed with the sample in the nanospray emitter by means of a microliter syringe.

In some cases, interfering signals from isobaric impurities necessitated the application of far higher resolutions up to 22,000 (10% valley definition) for the correct assignment of target molecule peak intensities. In these cases, where because of the high-resolution conditions, only weak signal intensities can be obtained, the addition of a mass calibrant was omitted to avoid ion suppression effects leading to even more decreased sensitivity. Consequently, data acquisition was performed in profile mode scanning magnetically from 350 to 360 amu at a scan speed of 30 sec/dec. About 150 spectra for each sample were acquired and averaged. Recalibration of the mass scale was done manually by assigning the theoretical values to signals of known components that had been identified by their CID product ion spectra prior to the experiment. The conditions for the acquisition of CID product ion spectra were essentially the same for low- and high-resolution precursor ion selection. The transfer ion optics were optimized for maximum transmission using the protonated molecular ion of senecionine *N*-oxide (reference sample) at $m/z = 352$. The same signal was subsequently used for optimization of the relevant ion trap parameters, i.e., multipole 1 and multipole 2 offset, multipole lens, trap offset, and mulitpole RF DAC by semiautomatic tune procedures of the instrument control software. CID spectra of the respective precursor ions were recorded with a parent isolation width of 3.0 amu and a normalized collision energy of 35% in normal centroid scan mode between 95 and 400 amu. Depending on precursor ion intensity, between 10 and 50 spectra had to be acquired and averaged subsequently with respect to the required signal-to-noise ratio of the resulting spectra. Total sample consumption was between 5 and 15 μl depending on the performed experiments.

Gas Chromatography – Mass Spectrometry (GC-MS). GC-MS data were obtained with an Hewlett-Packard 5890A gas chromatograph equipped with a 2-m fused silica guard column (deactivated, i.d. 0.32 mm) and a 30 m $\times$ 0.32 mm analytical column (ZB1, Phenomenex). The capillary column was directly coupled to a triple quadrupole mass spectrometer (TSQ 700, Finnigan). The analyses were under the following conditions. Injector and transfer line were set at 280°C; the temperature program used was 100°C (3 min)–300°C at 6°C min^{-1}. The injection volume was 1 μl. The split ratio was 1:20, the carrier gas flow was 1.6 ml min^{-1} He, and the mass spectra were recorded at 70 eV.

Quantitative gas chromatography was achieved by using a capillary column (15 m $\times$ 0.25 mm fused-silica; DB-1, J&W Scientific) (Witte et al., 1993). All other GC conditions were the same as given for GC-MS. Detectors were FID and PND. Quantitative analyses were performed *via* the FID signals by using heliotrine as internal standard.

RESULTS

Preparation of [^{14}C]Senecionine [^{18}O] N-*Oxide*. The senecionine applied as substrate contained approximately 10^7 cpm [^{14}C]senecionine and was prepared biosynthetically using *Senecio vulgaris* root cultures (Hartmann, 1994). The substrate was essentially free of seneciphylline. This is important because seneciphylline [^{18}O]*N*-oxide has the same molecular mass as unlabeled senecionine *N*-oxide and could interfere with MS analysis. The prepared 5.5-mg double-labeled [^{14}C]senecionine [^{18}O]*N*-oxide contained 96.5% ^{18}O in *N*-oxide oxygen, as established by ESI-MS and had a specific activity of $1.94 \cdot 10^8$cpm/mmol. The double-labeled tracer was radiochemically and chemically pure. An accurate mass measurement confirmed the correct elemental composition ($[M+H]^+$: $C_{18}H_{26}NO_5^{18}O$; found 354.1812 Da S. D.: 0.4 mDa; calculated: 354.1797 Da). CID product ion spectra were recorded as a reference for latter identification by comparison. No contamination with other PAs could be detected by high-resolution ESI-MS and GC-MS of the Zn/H^+-reduced sample.

Uptake and Storage of [^{14}C]Senecionine [^{18}O] N-*Oxide by* Oreina cacaliae *and* O. speciosissima. From feeding studies with radioactively labeled PAs, it is well-documented that the two PA sequestering leaf beetles *O. cacaliae* and *O. speciosissima* are able to absorb PA *N*-oxides and transfer them into their defensive secretions (Rowell-Rahier et al., 1991). Moreover, *Oreina* is unable to *N*-oxidize tertiary PAs (Hartmann et al., 1999). The recovered material upon ESI-MS almost exclusively exhibited the expected signals for ^{18}O-labeled and unlabeled senecionine-*N*-oxide (Figure 2). The CID product ion spectra of the signals at $m/z = 352$ ($[M + H]^+$ senecionine [^{16}O]*N*-oxide) and 354 ($[M + H]^+$ senecionine [^{18}O]*N*-oxide) in the samples of *O. speciosissima* and *O. cacaliae* (not shown in the figure) proved to be identical with those obtained from the synthetic tracer. Thus, the degree of ^{18}O labeling was calculated directly from the signal intensities (Table 1). In *O. speciosissima*, analysis of the senecionine *N*-oxide in the defensive secretion revealed a recovery of 99.1% of the ^{18}O and 102.5% of specific activity. This indicates that the orally fed senecionine *N*-oxide was taken up unaltered and transferred from the hemolymph *via* the exocrine glands into the defensive secretion. The same applies for *O. cacaliae*. In this case, however, the recovery of ^{18}O was only 64.9%. Since the specific radioactivity is reduced to almost the same extent (68.8%), the reason is not loss of ^{18}O label from fed tracer but dilution by nonlabeled background alkaloid already present in the beetle's secretion.

Uptake and Storage of [^{14}C]Senecionine [^{18}O] N-*Oxide by* L. jacobaeae. The determination of the degree of ^{18}O labeling by a simple low-resolution ESI measurement as described for the other samples was not feasible since the CID product ion spectra of the precursor ion at $m/z = 354$ showed minor, but

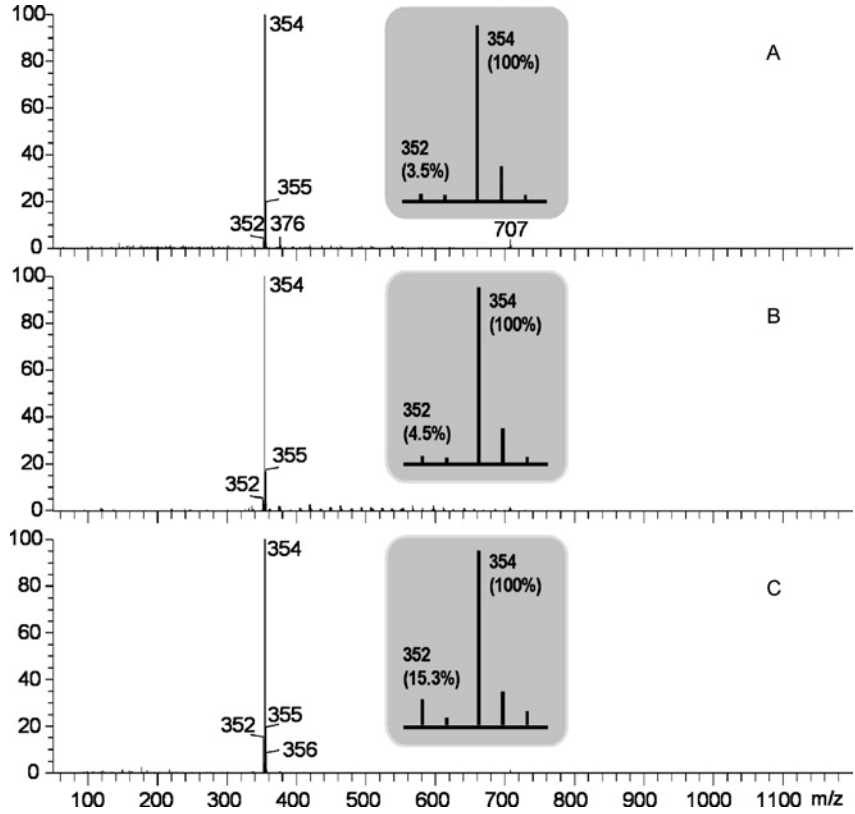

FIG. 2. Electrospray mass spectra (ESI-MS) of synthetic [^{14}C]senecionine [^{18}O]*N*-oxide (substrate) (A), senecionine *N*-oxide isolated from *O. speciosissima* defensive secretions (B), and from *L. jacobaeae* beetle extracts (C) after feeding of the double-labeled substrate.

TABLE 1. FEEDING OF [^{14}C]SENECIONINE [^{18}O]*N*-OXIDE TO *Oreina speciosissima* AND *O. cacaliae*

Sample	Ratio ^{16}O/^{18}O(%)	^{18}O recovered(%)	Specific radioactivity (cpm/mmol) ($\times 10^8$)	Specific radioactivity Recovered(%)
Tracer fed	3.5:96.5	100	1.94	100
Oreina speciosissima	4.4:95.6	99.1	1.99	102.5
Oreina cacaliae	37.4:62.6	64.9	1.37	68.8

Note. Analysis of the ^{16}O/^{18}O composition and specific radioactivity of senecionine *N*-oxide recovered from the defensive secretions.

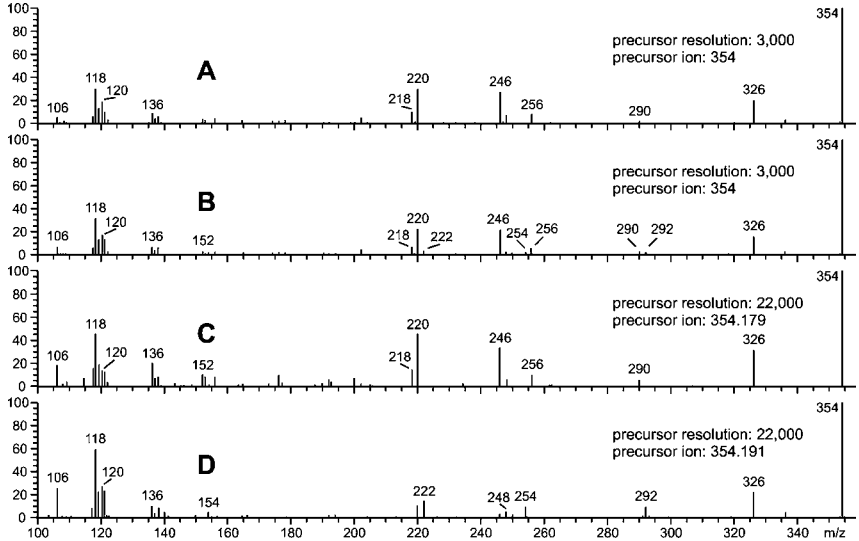

FIG. 3. CID product ion spectra of the senecionine *N*-oxide fraction from extracts of *Longitarsus jacobaeae* after feeding [^{14}C]senecionine [^{18}O]*N*-oxide: double-labeled substrate (A), purified beetle extract (B), major component from beetle extract (C), minor component from beetle extract (D) (see Figure 4.)

significant, differences compared to those of the tracer standard. Additional peaks at $m/z = 222$, 254, and 292 not present in the spectra of the standard material were evident (Figure 3). An accurate mass measurement (354.1853 Da S. D.: 0.3 mDa) showed a deviation from the theoretical value (354.1797 Da) exceeding the error limits that are typically valid for the instrument in this mode of operation, as can be seen from the value obtained for the tracer standard (354.1812 Da S. D.: 0.4 mDa). This suggested the presence of an isobaric impurity that coeluted in the preceding HPLC isolation. The appearance of a second component contributing to the signal at $m/z = 354$ was confirmed in a subsequent experiment with drastically increased resolution of 22,000 (10% valley definition) revealing a partially resolved doublet of peaks with a ratio of roughly 3:2 (Figure 4). Because of the low sensitivity in this mode, a further increase of resolution and the addition of a mass calibrant to enable accurate mass measurement by peak matching were not feasible. The achieved resolution and signal intensities proved sufficient for the successful detection of distinct CID product ion spectra of both components (Figure 3). The additional signals at $m/z = 222$, 254, and 292 mentioned above originated exclusively from the minor component, while the major component was unambiguously identified as senecionine [^{18}O]*N*-oxide by comparison with the reference data. This in turn allowed for the recalibration of the mass scale by assigning the correct

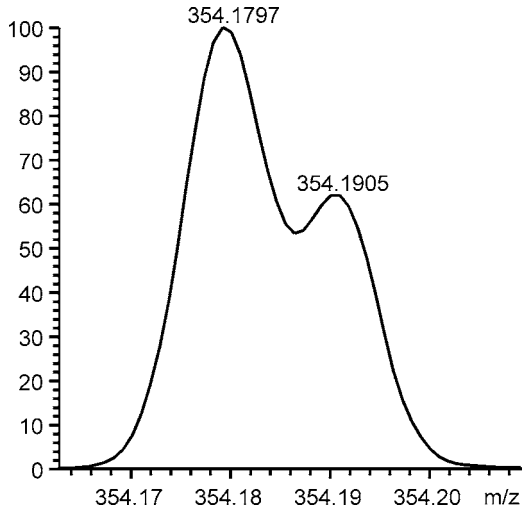

FIG. 4. High-resolution (22,000) ESI-MS of the signal at $m/z = 354$ from *L. jacobaeae* beetle extract showing a partially resolved doublet: left peak = senecionine [^{18}O]*N*-oxide, right peak = dihydrosenecionine [^{16}O]*N*-oxide.

mass value of 354.1797 Da ($C_{18}H_{26}NO_5^{18}O$) to the apex of the major component. Linear extrapolation, which should be valid for small mass increments, furnished an accurate mass of 354.1905 Da for the minor component. This suggested the presence of a hydrogenated congener of senecionine *N*-oxide (theoretical value $C_{18}H_{28}NO_6$: 354.1911 Da) they had not been separated by HPLC.

A computer-based simulation of the superimposed signals of the two compounds obtained by ESI, insured that the chosen resolution provided sufficient peak separation for the determination of the individual abundances from the peak maxima. Thus, the ratio of senecionine [^{18}O]*N*-oxide and the hydrogenated congener, assuming negligible differences in ionization efficiency, was determined to be 61%:39%.

By comparing the peak top intensities of the signals at $m/z = 352$ (senecionine [^{16}O]*N*-oxide; 35.0%) to the one at $m/z = 354$ (senecionine [^{18}O]*N*-oxide; 100%), the degree of ^{18}O labeling was determined to be 74%. A thorough comparison of the peak profiles at $m/z = 356$ (data not shown) obtained by the high-resolution experiment with simulated data revealed that the observed peak profile could not be explained solely by natural carbon-13 isotopic distribution of the tracer and the hydrogenated congener.

An additional component superimposed on the second carbon-13 isotopic peak of the hydrogenated congener was present. This most likely originates from a small amount of ^{18}O-labeled hydrogenated congener.

Because of insufficient signal intensity and resolution, a further characterization of this component by accurate mass determination or product ion scans was not possible. The abundance of this compound was estimated to be below 8% relative to the unlabeled hydrogenated congener.

To obtain more information about the suspected hydrogenated congener, the *N*-oxides were reduced and analyzed by GC and GC-EI-MS. Two major components were observed in the corresponding chromatogram, one of which was unambiguously identified as senecionine by its retention index (RI 2294) and its mass spectrum (Figure 5A). The other component (RI 2275) showed an almost identical pattern of signals in the lower mass range: Three prominent triads of signals at *m/z* 138,137, and 136, 121,120, and 119; and 95, 94, and 93. These peaks represent the typical main fragments of 1,2-unsaturated pyrrolizidine alkaloids (Bredenkamp, 1990) (Figure 5B). In contrast, many of the fragments in the upper mass range, still containing parts of the necine acid moiety, showed an increase of two mass units (e.g., *m/z* 222 vs, 220, 250 vs, 248, 292 vs, 290, 322 vs, 320, 337 vs, 335). This strongly suggests that the unknown compound represents a dihydrosenecionine that still contains the 1,2-unsaturation, tentatively assigned as 15,20-dihydrosenecionine (Figure 5B).

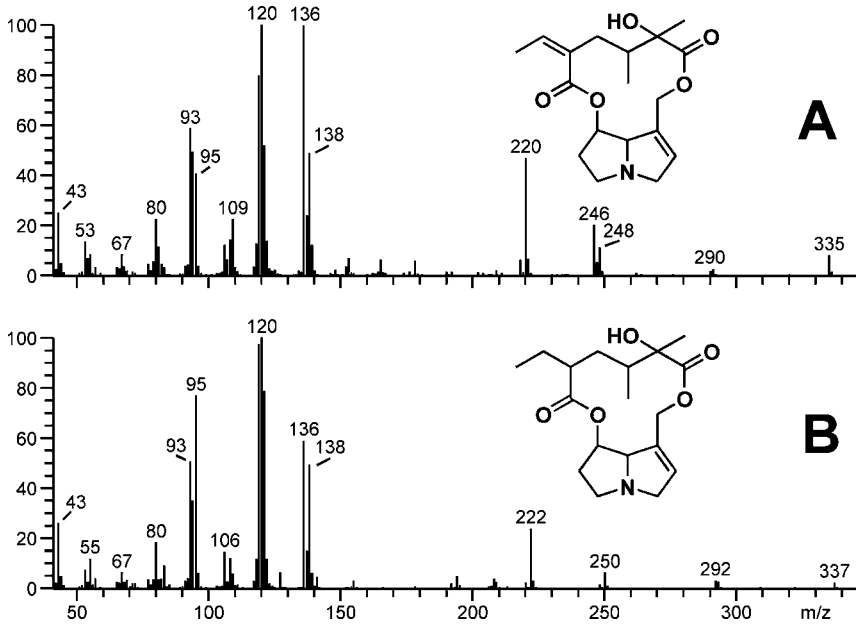

FIG. 5. EI mass spectra of the two main components recovered from *L. jacobaeae* beetle extracts after Zn/H^+ reduction: senecionine (A) and dihydrosenecionine (B).

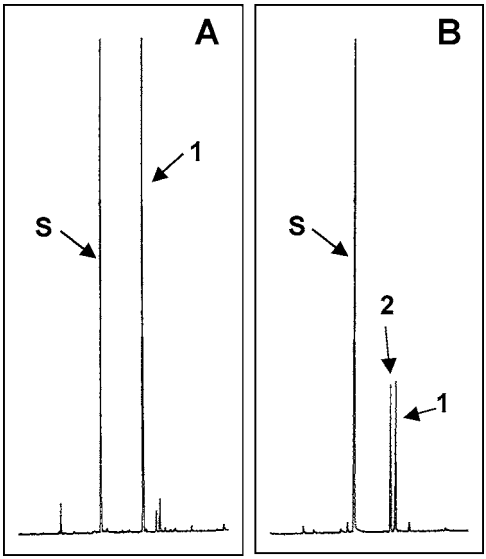

FIG. 6. Sections of GC profiles (column: DB-1, 15 m) of (A) the free base obtained from reduction of [^{14}C]senecionine [^{18}O]*N*-oxide fed as substrate to *Longitarsus jacobaeae* and (B) the free bases obtained from reduction of the HPLC-purified senecionine *N*-oxide fraction of beetle extract. S, heliotrine as internal standard; 1, senecionine (RI 2275); 2, dihydrosenecionine (RI 2248).

The GC analysis revealed a composition of 47% senecionine and 53% dihydrosenecionine (Figure 6). The differences in relative abundance of senecionine with respect to dihydrosenecionine in ESI MS vs. GC-MS may be explained by the different samples analyzed. In the case of ESI MS, one of the four replicates was analyzed, whereas for GC-MS, an aliquot of the combined replicates was analyzed.

In summary, the results of the *L. jacobaeae* experiment (Table 2) are as follows. (1) The HPLC-purified senecionine *N*-oxide fraction obtained from extracts of *L. jacobaeae* beetles previously fed with [^{14}C]senecionine [^{18}O]*N*-oxide revealed two compounds: senecionine *N*-oxide and dihydrosenecionine *N*-oxide. (2) Seventy-four percent of the senecionine *N*-oxide consisted of the ^{18}O-labeled *N*-oxide, whereas less than 8% of the dihydro derivative was labeled. (3) Most likely the unlabeled dihydrosenecionine *N*-oxide originates from the alkaloidal background of the beetles; only a small proportion of about 8% may be derived by hydrogenation of the labeled senecionine *N*-oxide fed. (4) The recovery of 77% ^{18}O in the senecionine *N*-oxide fraction, but no obvious dilution of the specific radioactivity, indicates that the lowered recovery of ^{18}O could not be due to

TABLE 2. FEEDING OF [^{14}C]SENECIONINE [^{18}O]*N*-OXIDE TO *Longitarsus jacobaeae*

Sample	Ratio ^{16}O/^{18}O(%)	^{18}O recovered(%)	Specific radioactivity (cpm/mmol) ($\times 10^8$)	Specific radioactivity Recovered(%)
Tracer fed	3.5:96.5	100	1.94	100
Senecionine *N*-oxide	26:74	77	2.21	113.8
Dihydrosenecionine *N*-oxide	ca. 92:8	<8		
Senecionine *N*-oxide + dihydrosenecionine *N*-oxide			1.03	53.2

Note. Analysis of the ^{16}O/^{18}O composition and specific radioactivity of the senecionine *N*-oxide fraction recovered from beetle extracts.

dilution by ^{16}O background *N*-oxide but must be caused by partial reduction of the fed [^{18}O]*N*-oxide and re-*N*-oxidation of the respective radioactively labeled senecionine. (5) The fact that 77% of the original ^{18}O-label of the tracer fed to the beetles is retained indicates that the intact *N*-oxide is absorbed and stored by the beetles.

DISCUSSION

Proof of a Carrier-Mediated Transport of PA N-Oxides. The feeding experiments with senecionine *N*-oxide ^{18}O-labeled in its *N*-oxide oxygen prove that *Oreina* and *Longitarsus* leaf beetles are able to absorb plant-acquired PA-*N*-oxides without preceding reduction. For *Oreina* this result confirms previous biochemical studies showing that plant-acquired PA-*N*-oxides build up a storage pool in the hemolymph and body tissues that serve to fill the defensive glands (Hartmann et al., 1997, 1999) where the concentration of PA *N*-oxides may reach levels of up to 0.3 mol·l^{-1} (Rowell-Rahier et al., 1991). *Oreina* leaf beetles are not able to *N*-oxidize any absorbed tertiary PA (Ehmke et al., 1991), instead they convert tertiary PAs into glucosides, that are assumed to be detoxification products (Hartmann et al., 1999).

With *L. jacobaeae* the situation is more complex. In this study, the main proportion (>75%) of the orally applied ^{18}O-labeled senecionine *N*-oxide accumulated unaltered in the beetle. The remaining portion of <25% accounted for ^{16}O-*N*-oxide. Since the specific radioactivity indicates that no unlabeled background senecionine *N*-oxide was present in the beetles, this fraction must be derived from re-*N*-oxidation of radioactively labeled senecionine produced by reduction of the ^{18}O-*N*-oxide.This corroborates previous studies where we showed that radioactively labeled senecionine is *N*-oxidized when fed orally or injected into the beetles' hemolymph (Narberhaus et al., 2003). Thus, in comparison to

Oreina, L. jacobaeae is not only able to directly sequester the PA *N*-oxides, but also able to detoxify any tertiary PA by *N*-oxidation.

To be sequestered, plant-derived PAs have to cross cellular barriers. While tertiary alkaloids passively diffuse through biological membranes, PA *N*-oxides are hydrophilic, salt-like substances that are unable to do so unless a specific membrane carrier is present. This study suggests the presence of such PA membrane carriers in the beetles investigated. In all three species, the gut epithelium has to be crossed to enter the body. In the case of the *Oreina* species, a second passage is necessary to enter the secretory gland cells (Figure 7B). In *L. jacobaeae*, defensive secretions could not be detected. However, storage organs must be present that are inaccessible to the *N*-oxygenase according to our earlier long-term storage experiments with this species. These analyses showed that a stable proportion of tertiary PAs was stored over prolonged time periods without being *N*-oxidized, thus suggesting a transient *N*-oxidation of PAs in the beetles' hemolymph (Narberhaus et al., 2004) (Figure 7A).

The Different Strategies of Handling PAs in Adapted Insects. We conclude that both *Longitarsus* and *Oreina* are equipped with specific, but partly different, key adaptations to PAs. Both may absorb plant-derived PA-*N*-oxides directly *via* carrier-mediated membrane transport and store the *N*-oxides in their bodies (Figure 7). In the case of *Oreina*, there is a second specific membrane transfer from the hemolymph *via* the cells of the exocrine glands into the defensive secretions (Figure 7B). Since the PA *N*-oxides are concentrated in the defensive secretions, transport into the glands must be an energy-dependent active transport counteracting a steep concentration gradient (Hartmann et al., 1999). Whether the carrier-mediated transfer of PA *N*-oxide from the gut into the hemolymph is active or passive is unknown.

The major difference between *Oreina* and *Longitarsus* is how they handle tertiary PAs. PA-*N*-oxides are easily reduced even in presence of weak reducing agents such as cysteine (Hartmann and Toppel, 1987). Orally ingested PA *N*-oxides are spontaneously reduced in the guts of most vertebrates and absorbed passively as lipophilic tertiary alkaloids (Mattocks, 1986). The same seems to be the general rule in insects, too. For instance, in *Spodoptera littoralis*, a noctuid moth that tolerates, but does not sequester PAs, ingested PA *N*-oxides are reduced and the resulting tertiary alkaloids absorbed. Nevertheless, *S. littoralis*, is able to tolerate PAs, in its food because it efficiently eliminates the absorbed protoxic tertiary PAs from the hemolymph back into the gut (Lindigkeit et al., 1997). PA-adapted arctiids and the African grasshopper *Z. variegatus* behave like *S. littoralis*, but instead of eliminating the absorbed protoxic PAs, they detoxify them by efficient and specific *N*-oxygenation, the resulting nontoxic *N*-oxides remain trapped in the body (Lindigkeit et al., 1997). *N*-oxide reduction and passive absorption, thus, appears to be the normal fate of ingested PA *N*-oxides in

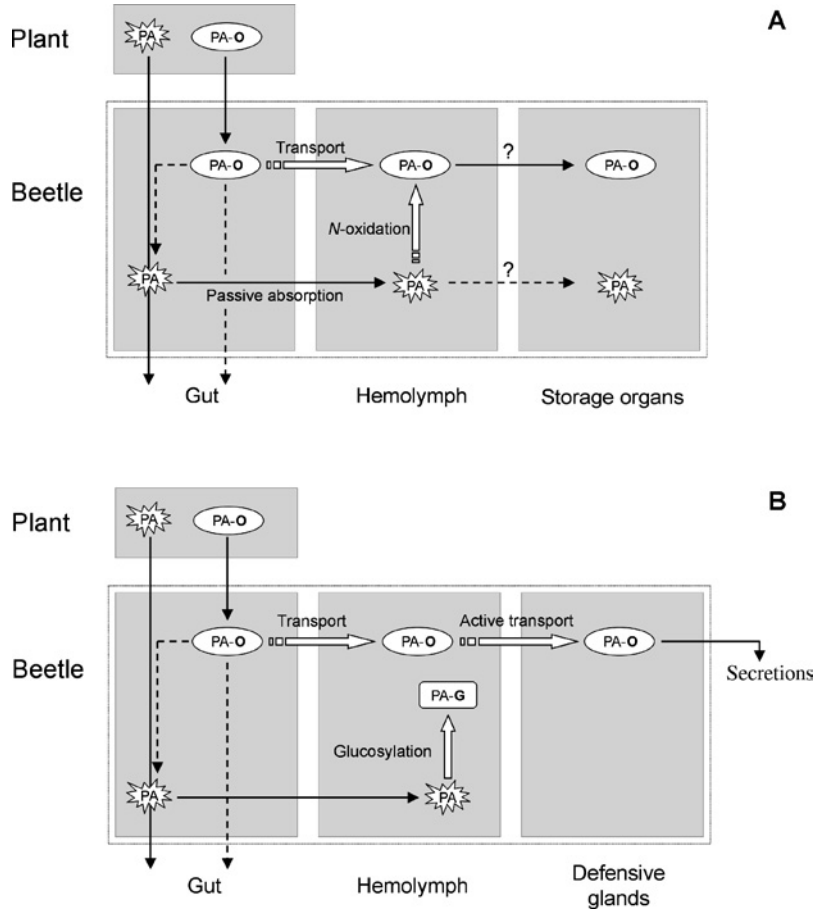

FIG. 7. Deduced PA pathways in *Longitarsus jacobaeae* (A) and *Oreina cacaliae* (B). Solid arrows: main pathways, dotted arrows: minor pathways. Thick white arrows: PA transport and specific metabolization. PA, tertiary pyrrolizidine alkaloid; PA-O, pyrrolizidine alkaloid *N*-oxide; PA-G, pyrrolizidine alkaloid glucoside.

most animals. In contrast, as demonstrated, *Oreina* and *Longitarsus* are both able to suppress the *N*-oxide reduction in the gut and absorb the plant-acquired PAs as nontoxic *N*-oxides. However, any protoxic tertiary PA formed in the gut or body is efficiently detoxified, either by *N*-oxidation, as confirmed for *Longitarsus* (Narberhaus et al., 2003), or by glucosylation as demonstrated for *Oreina* (Hartmann et al., 1999).

In addition to the different modes of detoxification of tertiary PAs, there is another striking difference between the two beetles. *Oreina* is unable to utilize tertiary PAs (Ehmke et al., 1991), whereas *Longitarsus* sequesters tertiary PAs with almost the same efficiency as *N*-oxides (Narberhaus et al., 2003). In that case, most of the alkaloids are stored as *N*-oxides by autogenous oxidation, while small amounts are retained as tertiary PAs in unknown compartments where they are no longer accessible to the *N*-oxygenase (see above; Figure 7A). Apparently, the suppression of reduction of ingested PA *N*-oxides is less efficient in *Longitarsus* than in *Oreina*. This is indicated by the substantial proportion of recovered senecionine *N*-oxide that has lost the ^{18}O label in the double tracer feeding experiment (i.e., about 23%; see Table 2). Thus, it appears that in *Longitarsus*-reduced PAs are recycled by re-*N*-oxidation whereas in *Oreina* the suppression of reduction appears to be so efficient that a "recycling" of tertiary PAs may be dispensable.

The results presented here complete our view regarding the strategies of PA-adapted insects to absorb, maintain, and store PAs and prevent self-poisoning. In any case, an accumulation of detrimental concentrations of protoxic tertiary PAs in metabolically active tissues is avoided. Presently, the following strategies can be documented. (i) Arctiid moths: the passively absorbed tertiary PAs are specifically *N*-oxidized and always maintained in the state of the nontoxic *N*-oxides. (ii) *Oreina* leaf beetles: the reduction of ingested *N*-oxides is efficiently suppressed and the *N*-oxides are absorbed *via* specific carrier-mediated transport and build up a storage pool in the body that serves to fill the exocrine glands; *Oreina* is unable to *N*-oxidize tertiary PAs that are, in contrast, detoxified by glucosylation. (iii) *Platyphora* leaf beetles: the passively absorbed tertiary PAs are efficiently removed from the hemolymph and pumped into the exocrine glands; no PA storage is found in the body outside the glands; *Platyphora* is unable to *N*-oxidize tertiary PAs. (iv) *Longitarsus* leaf beetles: the reduction of ingested *N*-oxides is suppressed and the *N*-oxides are absorbed *via* specific carrier-mediated transport; passively absorbed tertiary PAs are partly recycled into the *N*-oxides by specific *N*-oxidation and partly stored as tertiary PAs in a compartment where they are apparently separated from the metabolically active tissue. Thus, *L. jacobaeae* combines the basic strategies of the other three PA-adapted insect taxa: It is able to *N*-oxidize tertiary PAs, specifically absorb the *N*-oxides and safely store protoxic tertiary PAs.

Is Dihydrosenecionine a Specific Metabolite of L. jacobaeae ? A specific metabolic ability of *L. jacobaeae* was observed by chance: the presence of a dihydrosenecionine *N*-oxide, tentatively identified as the 15,20-dihydro derivative, that as an unexpected hydrogenated congener of the senecionine [^{18}O] *N*-oxide caused some complications in the ^{18}O-tracer experiment. Dihydrosenecionine is already known from the PA profile of a *L. jacobaeae* field population (Dobler et al., 2000). It has, however, not been found in the leaves of the beetle's food plant nor has it ever been reported to occur in *S. jacobaea*, which is one of the best studied *Senecio* species (Witte et al., 1992). Thus, it is likely that the

beetles themselves catalyze the hydrogenation of plant-acquired senecionine. In our feeding experiment of *L. jacobaeae* with senecionine [^{18}O]*N*-oxide, the direct MS analysis suggested a proportion of up to 8% double-labeled molecules in the recovered dihydrosenecionine *N*-oxide (Table 2). The hydrogenation of a small proportion of labeled senecionine *N*-oxide is also evidenced by a comparison of the specific activities of the recovered reduced PAs (Table 2). However, the efficiency of senecionine hydrogenation in adult beetles appears to be low although dihydrosenecionine is generally dominating in field-collected control samples. Therefore, the substance might possibly originate in larval metabolism. Only tracer experiments with larvae might clarify this issue.

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ENVIRONMENTAL AND ONTOGENETIC CONTROL OF ACCUMULATION OF BRACHYCERINE, A BIOACTIVE INDOLE ALKALOID FROM *Psychotria brachyceras*

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Abstract—Brachycerine is a monoterpenoid indole alkaloid accumulated in *Psychotria brachyceras* plants (Rubiaceae). To better understand the accumulation patterns of this alkaloid, we investigated its content in different plant organs from field-grown trees, throughout the seasons, during seedling development, and in response to potential biotic factors regulating its biosynthesis. Quantification by RP-HPLC showed that aerial vegetative organs (green stems, young and old leaves) yielded similar amounts of brachycerine [0.1–0.2% dry weight (DW)]. Brachycerine was not detected in roots. In reproductive structures, the highest brachycerine amounts (0.3% DW) were found in inflorescences. Alkaloid concentration decreased in mature fruits (0.045% DW). The lowest concentration in reproductive organs was observed in quiescent seeds (0.004% DW). Apparently, brachycerine content dropped during radicle emission in germinating seeds. During seedling development, an increase in leaf content from 0.02 to 0.1% DW was observed between the stages of 2 and 14 leaves, respectively. Salicylic acid did not affect brachycerine content. A doubling of

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alkaloid content was observed in wounded plants, and a threefold induction occurred with jasmonic acid treatment, suggesting that brachycerine biosynthesis is regulated by jasmonate production.

Key Words—Monoterpenoid indole alkaloid, herbivory, wounding, jasmonic acid, salicylic acid, developmental control, seasonal pattern.

INTRODUCTION

Plants synthesize a broad range of secondary metabolites that are often under strict developmental regulation. Some of these compounds are produced in response to environmental stresses, such as pathogen attack, wounding, and UV radiation, and can act as a defense mechanism (Facchini, 2001; Ramachandra Rao and Ravishankar, 2002; Gregianini et al., 2003). Alkaloids play several roles in plants due to antimicrobial, feeding deterrent, or allelopathic properties. Environmental factors play a fundamental role in the control of development and secondary metabolism (St-Pierre et al., 1999). Studies using *Catharanthus roseus* as a model system have shown that the biosynthesis of terpenoid indole alkaloids could be regulated by biotic and abiotic stimuli, and may be activated at particular stages of plant development (reviewed in Facchini, 2001).

Psychotria brachyceras Muell. Arg. (Rubiaceae) grows as a shrub (1–3 m in height) and is widely distributed in tropical and subtropical forests of Brazil (Smith and Downs, 1956), ranging from the state of Rio de Janeiro to Rio Grande do Sul (Dillenburg and Porto, 1985). Brachycerine (Figure 1) is the main alkaloid produced by this species and is probably derived from tryptophan, representing a new class of indole alkaloids. Its structure suggests a direct condensation of tryptamine with 10-oxo-1-*epi*-loganin (Kerber et al., 2001) instead of secologanin, and the subsequent formation of strictosidine, the general precursor of all known terpenoid indole alkaloids. Brachycerine has antinflammatory activity in a chemotaxis assay (A. Henriques, unpublished data), and an ethanolic extract of *P. brachyceras* leaves had non-specific analgesic activity (Elisabetsky et al., 1997). Brachycerine is produced in shoots and is absent in roots and undifferentiated tissue; moreover, it is strongly induced by UV exposure in leaves of *P. brachyceras* cuttings and, *in vitro*, the alkaloid was able to quench singlet oxygen (Gregianini et al., 2003). Brachycerine accumulation was not induced by various concentrations of hydrogen peroxide or the herbicide paraquat, a generator of superoxide anion (Gregianini and Fett-Neto, unpublished results).

The content of secondary metabolites may be enhanced following herbivory and mechanical damage in several plants (Kessler and Baldwin, 2002). Jasmonic acid (JA) and Salicylic acid (SA) are two endogenous signals implicated in eliciting plant resistance responses (Schmelz et al., 1998). SA seems to be synthesized from phenylalanine by benzoic acid hydroxylation (León et al., 1993) or from chorismate via isochorismic acid in plastids (Métraux, 2002). JA is a terminal

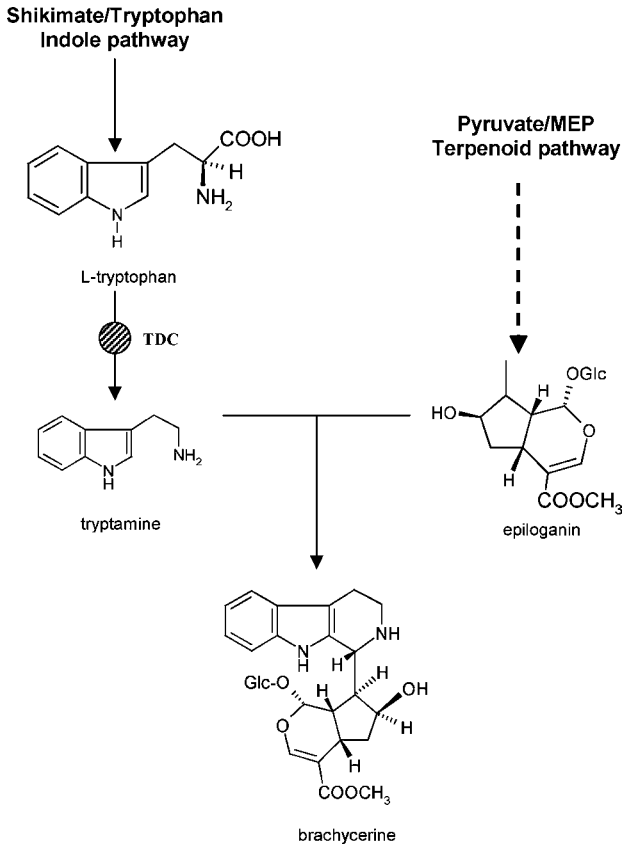

FIG. 1. Putative brachycerine biosynthetic pathway. The indole unit is probably derived from the amino acid tryptophan, which is converted to tryptamine by the cytosolic enzyme tryptophan decarboxylase (TDC). The terpenoid moiety is provided by isopentenyl diphosphate (IPP) or dimethylallyl diphosphate (DMAPP) via the plastid triose phosphate/pyruvate pathway (Contin et al., 1998). MEP, 2-*C*-methyl-*D*-erythritol-4-phosphate.

product of the octadecanoid pathway derived from membrane lipid catabolism; it is involved in wounding responses, pathogen attack, and in signal transduction of elicited secondary metabolite production (Farmer and Ryan, 1992; Rhodes, 1994; Schmelz et al., 1998; Devoto and Turner, 2003). The induced accumulation of JA and SA activate a variety of insect and microbial defense responses in plants, which may culminate in alkaloid production.

A detailed analysis of the dynamics of accumulation of brachycerine in *P. brachyceras* is an important step for developing sustained supply systems aiming

at pharmaceutical applications. Moreover, the identification of potential *in planta* functions for brachycerine may reveal other important properties of this molecule.

This report describes the distribution and accumulation of brachycerine in different vegetative and reproductive organs of field-grown *P. brachyceras* trees, seasonal patterns of alkaloid accumulation in leaves, and changes throughout early stages of development. Moreover, alkaloid content in leaves was examined upon challenging with wounding and signaling factors (i.e., JA and SA) potentially involved in the regulation of secondary metabolism.

METHODS AND MATERIALS

Plant Material. *Psychotria brachyceras* Müll Arg. (Rubiaceae) adult trees grown at Morro Santana – UFRGS, Porto Alegre, Rio Grande do Sul, Brazil, were used in the experiments.

Organ-Specific Distribution. The brachycerine content of different adult plant organs from field-grown trees was determined. For brachycerine distribution and concentration analysis, roots, young leaves (14.75 ± 2.25 and 47.4 ± 6 mm in width and length, respectively), and old leaves (31.6 ± 5.5 and 87.5 ± 12 mm in width and length, respectively) were collected on April 2003. Inflorescences were harvested on July 2002, stems and fruits on May 2001, and seeds on April 2002. Three replicates each with three different plants were harvested for every treatment.

Seasonal Analysis. Ten field-grown individuals from a natural stand were labeled and leaf samples (old leaves) were taken at every season for two consecutive years (2001 and 2002) at the same circadian phase of the day (mid-morning). Each plant represented one block and three replications per block were collected. Climatic data was obtained from the Instituto Nacional de Pesquisas Espaciais (INPE) and Instituto Nacional de Meteorologia (INMET) at Porto Alegre – RS, Brazil.

Seedling Development. Mature fruits were collected on April 2002. Seeds were surface sterilized with 70% (v/v) ethanol for 1 min, followed by immersion in 2% (v/v) sodium hypochloride with a few drops of detergent for 15 min. Seeds were cultured in 0.1 × MS medium (Murashige and Skoog, 1962) containing 6 $g \cdot l^{-1}$ agar (E. Merck, Darmstadt, Germany) under white fluorescent light with a photoperiod of 16 hr (approximately 35 $\mu mol.m^{-2}.sec^{-1}$) at $25 \pm 3^{\circ}C$. Samples were collected in five stages of development: quiescent seeds, imbibed seeds, germinated seeds (visible radicle), 2-leafed seedling, and 14-leafed seedling. Harvested samples were immediately placed into liquid nitrogen and frozen until analysis of alkaloid content.

Clonal Propagation for Elicitation Assays. Tip cuttings obtained from field-grown trees were cultured for 60 days in a solution of 0.1 × MS salts after an initial 7-day-exposure to 10 $mg.l^{-1}$ auxin, 4-(-3-indolyl) butyric acid (IBA, Sigma

Chemical Co, St. Louis, MO), in order to induce rooting (Kerber et al., 2001). Alternatively, rootless cuttings were directly incubated for 10 days in a solution of 0.1 × MS salts for adaptation prior to the experiments. In all elicitation treatments, incubation was done in a growth room with a photoperiod of 16 hr of white light (P.A.R. of approximately 73 μmol.m^{-2}.sec^{-1}) at 25 ± 3°. Leaves were harvested for alkaloid quantification at the beginning of the experiments and 2, 4, and 6 days after treatment application. Three replicates were used per treatment, and each replicate consisted of three plants.

Elicitation Assays. Wounding – Mechanical damage was applied with tweezers and scissors to approximately three-fourth of the total leaf area of cuttings and compared with intact cuttings. Both damaged and intact leaves were harvested in treated cuttings. Stems were also harvested to investigate the possibility of alkaloid translocation. In a separate experiment, using the same wounding method, damage was applied to specific leaf pairs or individual leaves in different sections of the cuttings. Some cuttings were damaged in apical leaves; others, in basal ones. Undamaged leaves were extracted separately from wounded ones in order to investigate a possible systemic response. A third treatment involved damaging a leaf and analyzing the nearest leaf in the opposite side of the stem. For leaf wounding assays, a more detailed time course experiment was carried out to measure brachycerine content 2, 8, 12, 24, 36, and 48 hr after treatment.

Jasmonic Acid – Leaves of hydroponically grown cuttings were mildly scarified with sandpaper and inoculated with 5-μl drops (five drops per leaf) of 40 and 400 μM of JA (Sigma) dissolved in 50% (v/v) ethanol. Control plants were identically treated and inoculated with 5-μl drops of 50% ethanol only.

Salicylic Acid – MS salts-grown cuttings were transferred to solution containing 0.72 mM or 1 mM of SA (Merck KGaA, Darmstadt, Germany); 0.5 g.l^{-1} 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer (Merck KGaA, Darmstadt, Germany), pH 5.7, was used as an adjuvant (Vestena et al., 2001). Shoots were also sprayed once with 1 mM or 2 mM SA, followed by covering with transparent plastic film for 24 hr. Control plants were kept in MS salts with 0.5 g.l^{-} MES pH 5.7 and sprayed once with distilled water.

Brachycerine Extraction and HPLC Analysis. Alkaloid extraction and analysis were performed as previously described (Kerber et al., 2001; Gregianini et al., 2003). In short, approx. 1 g of plant tissue was extracted in methanol (HPLC grade), mixed, and ultrasonicated for 30 min, centrifuged at 5000× *g* for 10 min, and the supernatant was recovered. Chemical analysis was performed using a Waters Alliance 2690 HPLC system with photodiode array detector (PDA). Chromatography was performed on an Hibar RP-8 column (E. Merck, Darmstadt, Germany), using a linear gradient with methanol–water–trifluoroacetic acid. Eluted compounds were monitored at wavelengths between 200–400 nm with a Waters Millenium (version 2.15.01) diode array detector. Quantification was obtained using an external standard curve; identity and purity were based on retention time, UV-spectrum, and

co-chromatography with authentic brachycerine isolated from leaves. Brachycerine content is expressed on an extracted dry weight basis (DW).

Statistical Analysis. Statistical analysis of brachycerine content (in triplicates) was performed by simple or factorial (Treatment × Time) ANOVA followed by Duncan test at $P \leq 0.05$. Percent data were square root transformed (Sokal and Rohlf, 1981). All elicitation treatments had three replicates per treatment, and each replicate consisted of three plants. All experiments were independently repeated 2 to 3 times.

RESULTS AND DISCUSSION

Organ-Specific Alkaloid Distribution. Brachycerine distribution varied with organ type (Figure 2). Stems and leaves (both young and old) had similar amounts of brachycerine (0.1 to 0.2% DW) and were comparable to the concentration of catharanthine (0.2% DW), another terpene–indole alkaloid in *Catharanthus pusillus* leaves (Zárate et al. 2001). Brachycerine was not detected in roots, in agreement with Kerber et al. (2001). Inflorescences contained the highest brachycerine content (0.3% DW); in mature fruit pulp much lower amounts (0.045% DW) were

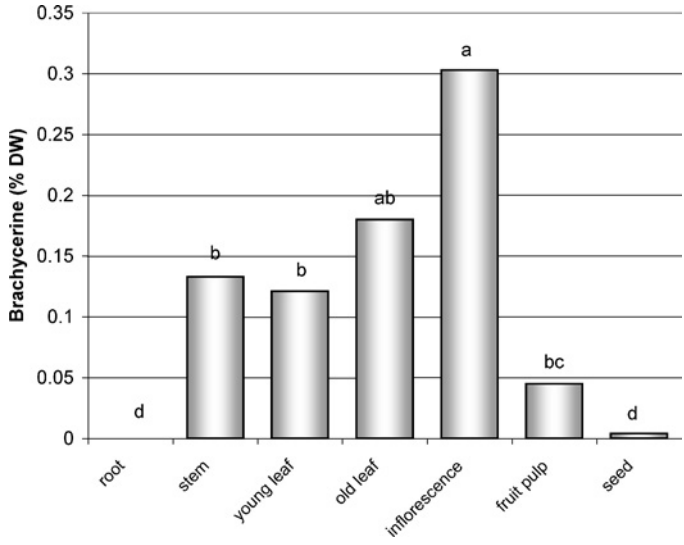

FIG. 2. Brachycerine distribution in different plant organs from *P. brachyceras* adult trees. Vegetative organs: root, stem, young, and old leaves. Reproductive organs: inflorescence, mature fruit pulp, and seed. Treatments sharing a letter are not significantly different at $P \leq 0.05$ by a Duncan test. Values represent the mean of at least three replicates.

observed (Figure 2). The higher accumulation in flowers may reflect a herbivory-deterrent role for the alkaloid and contribute to the success of fertilization and seed set. The lowest concentration of brachycerine (0.004% DW) was detected in quiescent seeds (Figure 2); the dilution of alkaloid content during the transition from inflorescence to mature fruit may be the result of biomass gain during seed and fruit filling and/or retranslocation to vegetative parts. At the fruit stage, lower concentrations of brachycerine may facilitate biotic seed dispersal. Interestingly, *P. brachyceras* young leaves, which are known to be more active in tryptophan decarboxylase (TDC) activity and alkaloid accumulation (Fernandez et al., 1989; Zárate et al., 2001) in other species, did not differ from old leaves in brachycerine content. In fact, a trend toward preferential accumulation in old leaves was observed (Figure 2). Accumulation of camptothecin in *Camptotheca acuminata* is higher in younger leaves and trees, which has been attributed to chemical defense of these nutrient-rich and tender parts (Liu et al., 1998). However, potential defense metabolites other than brachycerine may be present in young leaves of *P. brachyceras*.

Seasonal Analysis. Leaves were sampled from 10 different trees over two consecutive years (spring/2000–winter/2002). There was a seasonal effect on brachycerine content in the Morro Santana population. Concentrations were higher during the spring, fall, and winter from the first year and lower during the summer, independent of the analyzed individual. However, brachycerine remained constant and at lower levels throughout the second year, independent of the season evaluated (Figure 3). Leaves harvested during the second year (corresponding to stable and lower values of brachycerine) were exposed to slightly higher and more stable precipitation values (141.6 ± 41 mm vs. 133.6 ± 78.4 mm from the first year). In spite of the nonvolatile character of alkaloids, similar seasonal responses have been observed for monoterpenes in *Juniperus* from two areas in

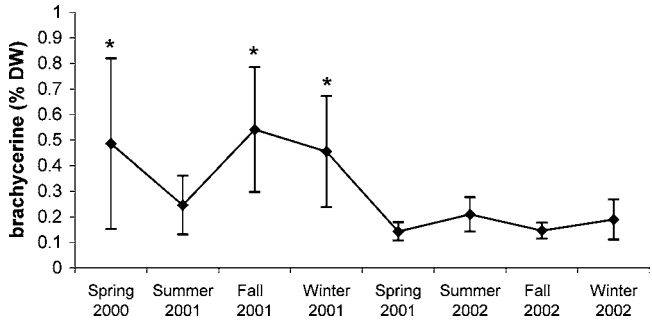

FIG. 3. Seasonal concentrations (% DW) of brachycerine in *P. brachyceras* leaves at Morro Santana. Treatments indicated by * are significantly different at $P \leq 0.05$ by a Duncan test. Values are means (±standard deviations) from three independent determinations.

Texas exposed to different precipitation rates (Owens et al., 1998). Some of the examined *P. brachyceras* individuals were consistently higher (e.g., 0.74 ± 0.25% DW) or lower (e.g., 0.25 ± 0.02% DW) accumulators of brachycerine independent of the time of year, which indicates a genetically based control of alkaloid accumulation.

Seasonal variations in other alkaloids have been reported and are complex. *Uncaria tomentosa* has been shown to accumulate higher amounts of alkaloids in the spring and summer and decrease in the fall, although these observations were restricted to young leaves (Laus et al., 1997). Moreover, some of the alkaloids evaluated (e.g., pteropodine 1, speciophylline 3, and uncarine F4) showed considerable differences in leaf content in the same plant at the same month over successive years.

Alkaloid composition and concentration of a species is at least partly under genetic control. Environmental conditions, which vary seasonally, such as light, drought stress, soil moisture, and fertility, may significantly modify the expression of alkaloid metabolism (Levin, 1976).

Seedling Development. The brachycerine content in germinating seeds tended to decrease during radicle emergence, suggesting metabolic modifications of the alkaloid or leakage into the medium. During seedling development, an increase in shoot alkaloid content was observed. Very young seedlings with a pair of cotyledons and two leaves yielded 0.02% DW brachycerine, reaching the amount found in field-growth plants (0.1% DW) at the 14-leaf stage (Figure 4). This higher content in mature seedlings may reflect biosynthesis resulting from photosynthetic metabolism (monoterpene moieties arise from the plastidic terpene pathway); the alkaloid could be involved in seedling protection against predators. Studies with germinating seedlings in other species have suggested that alkaloid

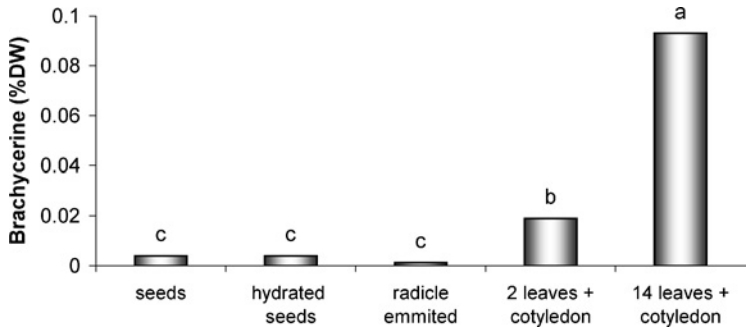

FIG. 4. Brachycerine content (% DW) during seedling development. Treatments sharing a letter are not significantly different at $P \leq 0.05$ by a Duncan test. Values represent the mean of at least three replicates.

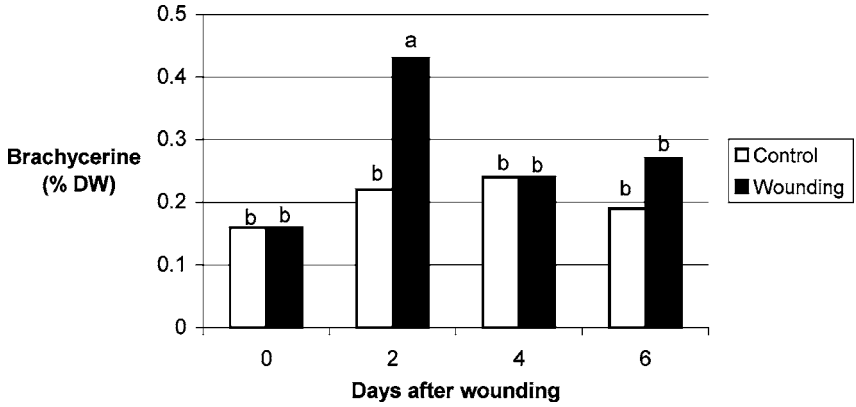

FIG. 5. Effects of wounding on brachycerine leaf content (% DW). White bars represent intact cuttings and dark bars wounded cuttings. Leaf samples were collected 2, 4, and 6 days after treatment. Treatments sharing a letter are not significantly different at $P \leq 0.05$ by a Duncan test. Each value represents the mean of three replicates.

biosynthesis and accumulation are associated with seedling development and with dry matter increase (reviewed in Williams and Harrison, 1983; St-Pierre et al., 1999).

Wounding, JA, and SA. Mechanical damage to *P. brachyceras* leaves induced a twofold increase (0.43% DW) in brachycerine content within 2 days (Figure 5). A detailed time course analysis indicated that an increase in brachycerine was not evident 2 hr after damage but started 8 hr after wounding (data not shown). After this induction peak, the amount of brachycerine decreased to basal levels. Mechanical damage may induce responses that are restricted to the wound site or responses that are rapidly propagated throughout the plant. To investigate the possibility of a systemic alkaloid accumulation response induced by wounding, different cutting sectors were elicited using the same experimental procedure. Alkaloid induction was restricted to the wound site, not constituting a systemic wounding response. The pattern of induction differed with leaf age, confirming a trend toward preferential accumulation in older leaves in *P. brachyceras*. Damage to young, apical leaves, resulted in a nonsignificant 17% induction on the second day after treatment, compared to a significant 3.4-fold increase when damage was applied to older basal leaves. Leaf alkaloid increases were not due to alkaloid transport from the stem. Total content of damaged cuttings (stem + leaves) was consistently higher than that of control cuttings (approx. 2.5-fold). Alkaloid content in individual stems was relatively stable and did not decrease in relation to leaves, whereas alkaloid amount in leaves increased significantly.

In contrast to *P. brachyceras*, the mechanical damage elicitation of *P. umbellata* cuttings, a closely related species, did not change umbellatine content in leaves (Paranhos and Fett-Neto, 2004, unpublished results) suggesting that this alkaloid is constitutive. In spite of the structural and chemotaxonomical relation among alkaloids from *Psychotria* species, these alkaloids show a distinct regulation in relation to wounding, probably representing examples of constitutive and inducible (at least partly) secondary metabolite accumulation. Indeed, basal leaf content of umbellatine in *P. umbellata* are approximately 10 times higher than those of brachycerine in *P. brachyceras*.

Indole alkaloid accumulation has been observed as a result of wounding stress in *Catharanthus roseus* (Frischknecht et al., 1987), although in this case alkaloid translocation was not excluded. In *Nicotiana sylvestris*, nicotine is synthesized *de novo* from (^{15}N)-nitrate in response to lesions in leaves; the alkaloid content reached is sufficient to reduce larval growth of the herbivore *Manduca sexta*. Simulated herbivory in leaves induced an alkaloid systemic response in *N. attenuata* (reviewed in Kutchan, 1995). In this species, wounding and mammalian herbivory increased nicotine production, which is in turn activated by proportional changes in endogenous JA production, as well as by exogenous JA applications (reviewed by Kessler and Baldwin, 2002).

In the present study, exogenous application of 40 μM jasmonate caused a 2.7-fold induction in brachycerine content 6 days after treatment, while 400 μM jasmonate increased the alkaloid content by 3.3-fold, 4 days after treatment (Figure 6). The continuous increase in alkaloid accumulation over time may be due to jasmonate mobility or activation of endogenous jasmonate production. These results suggest that the JA-signaling pathway operates in brachycerine metabolism. Brachycerine biosynthesis could be regulated by JA produced following cell damage and/or by exogenous jasmonate application. In *Eschscholtzia californica* cell cultures MeJA treatment resulted in a 5-fold increase in enzyme activity and a 12-fold increase in mRNA levels encoding for the berberine bridge enzyme, a key enzyme in isoquinoline alkaloid biosynthesis (Rhodes, 1994). In similar fashion, paclitaxel and its precursor baccatin III in *Taxus* sp. cell cultures were significantly increased by MeJA (Yukimune et al., 1996). Mechanical wounding and herbivory cause a coordinated transcriptional reorganization of the plant (Kessler and Baldwin, 2002) that is mediated by wound-specific *trans*-acting factors, such as *ORCA3,* a jasmonate responsive transcription factor from *Catharanthus roseus* that regulates in coordinated fashion the expression of various genes involved in the production of terpenoid indole alkaloids (Van der Fits and Memelink, 2000). The octadecanoid pathway also mediates responses to UV radiation (Hollósy, 2002), a stress factor that affects brachycerine biosynthesis, resulting in 10-fold increase in leaf concentration (Gregianini et al., 2003). Conconi et al. (1996) proposed a model to explain similarities between wounding and the UV response in activating wound-response genes. According to their model, UV radiation results

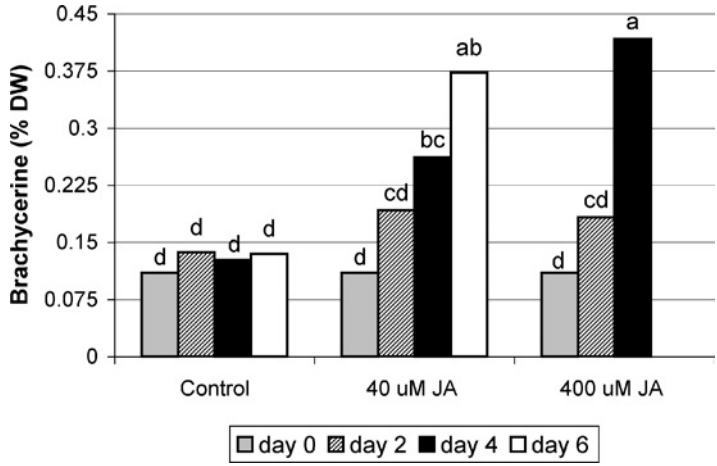

FIG. 6. Effects of jasmonic acid (JA) on brachycerine leaf content (% DW). Application of JA was done in 50% (v/v) ethanol on sand paper scarified leaves. Control cuttings were treated with 50% (v/v) ethanol applied on scarified leaves. Leaf samples were collected 2, 4, and 6 days after treatment with 40 μM, and 2 and 4 days after treatment with 400 μM JA. Treatments sharing a letter are not significantly different at $P \leq 0.05$ by a Duncan test. Each value represents the mean of three replicates.

in the perturbation of plant membranes and/or the activation of lipases that cause the release of linolenic acid, which engages the intracellular octadecanoid pathway to activate wound-inducible genes.

Contrary to the results of brachycerine induction by leaf wounding and JA application, SA did not affect brachycerine content at the concentrations used (data not shown). Similar results were found in *C. roseus* cell cultures where SA had weak induction effects on *Tdc* and *Str* steady-state mRNA levels (Pasquali et al., 1992), although SA has been shown to stimulate secondary metabolism in *C. roseus* cell suspension cultures (Godoy and Loyola, 1997). Similarly, exogenous methyl salicylate did not affect the accumulation of phytoecdysteroids in spinach (*Spinacia oleracea*) plants (Schmelz et al., 1998). These results suggest that brachycerine may be involved in plant defense, perhaps as a deterrent agent. Herbivore attack is frequently associated with wounding, and recognition of herbivory frequently involves modifications of a plant's wound response (Baldwin et al., 2001). JA and ethylene, which are involved in activation of wounding-responsive genes, mediate the signaling pathway of the wounding response and pathogenesis-related genes. SA is mainly associated with the establishment of systemic acquired resistance (SAR) and its levels increase after pathogen infection (Menke et al., 1999). Thus, some wounding pathway components may be involved in resistance to certain

pathogens and insects, since herbivory signal transduction cascade primarily uses JA and, secondarily, SA. Recent evidence indicates that signal pathways are not linear, but are integrated through a network of cross-talking connections to coordinate responses. Although there is substantial communication between the JA and SA pathways, JA and SA signal cascades activate different sets of plant defense genes (Thomma et al., 1998) or even act antagonistically (Felton et al., 1999). Depending on induction type, there may exist different control pathways or partial response superposition (Baron and Zambryski, 1995; McConn et al, 1997; Maleck and Dietrich, 1999).

The results indicate that brachycerine content is spatially and temporally regulated. Preferential accumulation in aerial parts and during the reproductive phase, as well as a reduction during germination and initial seedling growth, were observed. Seasonal variation in brachycerine content was observed with a lower accumulation in the summer, although not consistently in consecutive years. Individual variation in the capacity to accumulate the alkaloid was found with high and low producers maintaining regular accumulation profiles. The lack of increase in brachycerine content by exogenous SA suggests that this indole alkaloid has an action mechanism not predominantly related to general pathogen responses. Its role appears to be mainly associated with UV (Gregianini et al., 2003) and wound responses, probably mediated by JA.

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ENHANCEMENT OF ATTRACTION TO SEX PHEROMONES OF *Spodoptera exigua* BY VOLATILE COMPOUNDS PRODUCED BY HOST PLANTS

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Abstract—We measured the effects of exposure to volatile compounds produced by host plants on the rate of capture of male *Spodoptera exigua* using synthetic sex pheromones. Exposure to volatile compounds stimulated strong electroantennographic responses of male *S. exigua*. The behavioral responses of male moths to combinations of sex pheromone and volatile compounds were tested in wind tunnel experiments. When lures were baited with synthetic sex pheromone plus benzaldehyde, phenylacetaldehyde, (*Z*)-3-hexenyl acetate, or linalool, respectively, the landing rate of *S. exigua* males was increased by 101.4%, 79.6%, 60.6%, and 34.3%, respectively, compared to sex pheromone alone. In field tests, traps baited with either pheromone + (*E*)-2-hexenal, pheromone + phenylacetaldehyde, pheromone + (*Z*)-3-hexenyl acetate, or pheromone + (*Z*)-3-hexenol enhanced moth catches by 38.8%, 34.6%, 24.6%, and 20.8%, respectively compared to traps baited with pheromone alone. In a second field experiment, more *S. exigua* males were trapped with a combination of a synthetic sex pheromone blend and several individual host plant volatiles compared to synthetic sex pheromone alone. These results suggest that some host plant volatiles enhance the orientation response of *S. exigua* male moths to sex pheromone sources.

Key Words—*Spodoptera exigua*, sex pheromone, plant volatiles, behavior, wind tunnel, field trapping.

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INTRODUCTION

The beet armyworm, *Spodoptera exigua* (Hüber), is an important pest on a variety of crops in many areas of the world, including China. Beet armyworms are resistant to many insecticides and feed nocturnally, making control with conventional insecticides difficult (Moulton et al., 2000). Improvements in control methods for beet armyworms are of agricultural interest.

The female sex pheromone of beet armyworm was identified as a mixture of *Z*9*E*12-14: OAc, *Z*9-14: OH, *Z*9-14: OAc, and *Z*9*E*12-14: OH (Dong and Du, 2002). Synthetic pheromone of beet armyworm has been used in population forecasting (Suenaga and Tanaka, 1997), mass trapping (Kim et al., 1995), and mating disruption (Mitchell et al., 1997). However, as reducing pesticide application has become more important, methods that increase the effectiveness of attraction are of greater interest, and has led to searches for factors that enhance attraction.

Chemicals from host plants often enhance insect responses to sex pheromones (Landolt and Phillips, 1997). More *Heliothis zea* males were caught by a combination of synthetic female sex pheromone blend and either (*Z*)-3-hexenyl acetate or (*Z*)-3-hexen-1-ol compared to sex pheromone used alone (Light et al., 1993). More tobacco budworms (*Heliothis virescens*) were captured in traps baited with a combination of synthetic female sex pheromone and (*Z*)-3-hexenyl acetate than with pheromone alone (Dickens et al., 1993a). Similarly, male codling moths (*Cydia pomonella*) were trapped in a greater number when traps were baited with female sex pheromone and a blend of five compounds known to be green leaf volatiles in comparison to female sex pheromones alone (Light et al., 1993).

Here we selected several volatile compounds reported to be produced by host plants of the beet armyworm (Dickens et al., 1993b). We then tested whether these volatile compounds enhanced orientation responses of male *S. exigua* moths to female sex pheromones. Our goal is the design of an efficient lure for use in integrated pest management programs for *S. exigua*.

METHODS AND MATERIALS

Insects. Beet armyworm moths were obtained from a laboratory population at Nanjing Agricultural University (Nanjing, China), where two generations have been maintained since field collection. Larvae were reared in the laboratory at 25 $\pm$ 2°C under a reversed 14:10 hr light:dark photoperiod and fed on a modified, semiartificial diet as described by Li et al. (1991). After sexing, male pupae were placed in 40 cm $\times$ 25 cm $\times$ 15 cm wooden cages. Moths were fed on 10% sugar water solution saturated in cotton balls before testing.

Chemicals. (*Z*,*E*)-9,12-Tetradecadienyl acetate (*Z*9*E*12-14:OAc) was obtained commercially (Shin-Etsu Chemical Industries, Japan). (*Z*)-9-Tetradecen-1-ol (*Z*9-14:OAc) was synthesized and purified by column chromatography in

our laboratory. The purity of these compounds was measured using gas chromatography, and samples were found to be more than 95% pure. (*Z*)-3-Hexenyl acetate, (*Z*)-3-hexen-1-ol, linalool, and (*E*)-2-hexenal were provided by New England University of Australia (purity $\geq$ 98%). Benzaldehyde (purity $\geq$ 98.5%) and phenylacetaldehyde (purity $\geq$ 94%) were obtained commercially (Chinese Medical Chemical Company, Shanghai).

Wind-Tunnel Assays. The tests were performed in a Plexiglas wind tunnel, 250 cm $\times$ 96 cm $\times$ 96 cm under conditions of 25 $\pm$ 2°C, 70 $\pm$ 10% relative humidity, 0.3 lux (red light), and air speed of 30 cm/sec. Male moths in the third scotophase were tested because they had shown maximum response to female sex pheromone (Dong and Du, 2002). Before the onset of scotophase, males were transferred individually to test tubes (2.5 cm $\times$ 10 cm) and held until the 6th hr into scotophase. They were allowed to acclimate to tunnel conditions for 30 min, and then introduced into the tunnel individually. Filter paper containing the pheromone and/or host plant volatiles was pinned on a 20-cm high steel jack placed on the midline of the wind tunnel and 25 cm away from the upwind end. The steel jack with the lure was caged with a cylindrical screen cage (25-cm high, 10-cm in diam). For the test, a male moth was transferred from the test tube to a slightly larger cylindrical screen cage with one end open. Males were allowed to respond for 3 min, and scored for the following behaviors: takeoff (TF), orientation flight (OR), arrival at halfway (HW), approach to the cage containing the lure (within 10 cm) (AP), and landing on the cage containing the lure (LS).

Sex pheromone solution and host plant volatile solutions were prepared in redistilled hexane; the concentration of pheromone (*Z*9*E*12-14: OAc/*Z*9-14: OH=9/1) solution was 0.1 μg/μl for all experiments. A triangular piece of filter paper (5-mm base $\times$ 10-mm height) was used to present test solutions to males. 10 μl of hexane plus sex pheromone solution were applied to each filter paper prior to the bioassays. Lures were formulated by applying a solution containing 20 μg of a host plant volatile and allowing the solution to evaporate for 2 min. The lure was then pinned to a steel jack for testing. Each lure was used once. Responses of 10–17 males were recorded individually for each experiment with three replicates per formulation.

Electroantennography (EAG). EAG responses from isolated male antennae were recorded according to the method described by Du (1988). An antenna of a 2-day-old moth was excised from its base, and the distal part of the terminal segment was cut off. The antenna was mounted between two cotton balls, which were placed on the separated electrodes and saturated with Beadle-Ephrussi solution (Zhang and Meng, 2000). Air-carrier speed was kept at 1 m/sec. 10 μl of each solution containing 10-μg of the volatile to be tested were applied to a piece of filter paper (diam = 0.5 cm), and the filter paper was inserted into a glass syringe. A solution containing 1 μg of sex pheromone components (*Z*9*E*12-14: OAc/*Z*9-14: OH=9/1) served as a standard. An airstream of 1-sec duration was blown over the antennae. Intervals between stimuli were at least 2 min. Each

solution was tested with six antennae of males, and each antenna was tested three times.

Field Trapping. Attractiveness of synthetic sex pheromone (P) of *S. exigua* alone and/or its combination with host plant volatiles was evaluated in a Chinese cabbage field at Songjiang Horticultural Farm in Shanghai during October 8–24, 2001 when the field population of *S. exigua* began to decline. A second trapping experiment was conducted in a cotton field (Pengze County, Jiangxi province) during the peak emergence period of adult *S. exigua* (September 9–20, 2002).

Two types of trap dispenser were used. One was a red rubber septum impregnated with 200 μg of synthetic sex pheromone (*Z*9,E12-14: OAc/*Z*9-14: OH = 9/1). The second was a 600-μl plastic centrifuge tube containing host plant volatiles dissolved in 10-μl soybean oil. A 1-mm hole was bored in the cap of the tube when the trap was mounted in the field. A red plastic water pan (20-cm diam, 10-cm high) filled with dilute detergent solution to 8-cm deep was used as the trap.

The two different dispensers were hung close together 2 cm above the water surface of the trap. Traps were placed above 20-cm high in the Chinese cabbage field experiments. In the cotton field experiments, traps were placed about 10 cm above the plant canopy. Traps were placed ~20 m apart, with treatments randomly interspersed. Insects captured in the traps were removed daily, counted, identified, and sexed. Traps were re-randomized every 3 d in order to avoid any effect of trap location. Three replicates were used for each treatment.

Statistical Analyses. Analysis of variance (ANOVA) and least-significant difference (LSD) tests were used to analyze trapping data from experiments conducted in the Chinese cabbage field. ANOVA followed by Duncan's multiple range test was used to analyze EAG responses and data from the cotton field experiments. The distribution of insect responses from the wind tunnel bioassay was analyzed using a χ^2 test.

RESULTS

EAG Responses. Selected volatile compounds produced by host plants elicited significantly stronger EAG responses from the antennae of male *S. exigua* male moths when compared with the hexane control treatment (Table 1). There were no significant differences in responses among the compounds tested ($P > 0.05$).

Behavioral Responses. Behavioral responses of *S. exigua* males to different lures in the wind tunnel experiments varied significantly (Table 2). All males took flight, but responses differed among treatments for all other phases of lure location. Among the lures, P + phenyl acetaldehyde, P + benzaldehyde, and P + (*Z*)-3-hexenyl acetate elicited more types of behavior indicating attraction to the odor source and had the highest response rates at the AP and LS steps. The

TABLE 1. EAG RESPONSES OF ANTENNAE OF MALE *S. exigua* TO SEVERAL PLANT VOLATILES RELATIVE TO SYNTHETIC SEX PHEROMONE (Z9, E12-14: OAC/Z9-14: OH = 9/1)[a]

Chemical	EAG value ± SE (relative to P) (%)[b]
Phenylacetaldehyde	74.58 ± 6.03a
Benzaldehyde	72.67 ± 5.89a
Linalool	65.30 ± 4.86a
(*E*)-2-Hexenal	59.25 ± 7.11a
(*Z*)-3-Hexenol	61.24 ± 4.10a
(*Z*)-3-Hexenyl acetate	62.99 ± 6.51a
Hexane	25.95 ± 3.79b
P	100

[a] 1 μg of synthetic sex pheromone (P) and 10 μg of each plant volatile were tested.
[b] Percentage followed by a different letter are significantly different at $P < 0.05$ (Duncan's multiple range test).

response rate of all lures containing volatile compounds increased significantly at the LS step compared with that of the lure containing synthetic pheromone alone.

Field Trapping. In the field trapping experiment in a Chinese cabbage field, traps baited with synthetic sex pheromone in combination with some of the tested volatile compounds captured more moths than the traps baited with pheromone alone (LSD, $P < 0.05$) (Table 3). Traps baited with P + (*E*)-2-hexenal, P +

TABLE 2. BEHAVIORAL RESPONSES OF *S. exigua* MALES TO THE DIFFERENT LURES IN THE WIND TUNNEL[a]

Lure	No.	TF	Male response percentage (%)[b,c]			
			OR	HW	AP	LS
P	30	100	76.6	55.3	36.2	29.8
P + benzaldehyde	43	100	100***	90.0***	65.0***	60.0***
P + phenylacetaldehyde	40	100	100***	86.1***	53.5**	53.5***
P + (*Z*)-3-hexenyl acetate	48	100	91.3**	78.3***	50.0	47.8**
P + linalool	46	100	86.7	66.7	46.7	40.0
P + (*Z*)-3-hexenol	47	100	77.1	54.2	27.1	22.9

[a] In lures, the dosage of the synthetic sex pheromone (P) was 1 μg, and the dosage of each plant volatile added was 20 μg.
[b] Asterisks indicate differences (χ^2 test) from P: **, $P < 0.01$; ***, $P < 0.001$.
[c] TF = take off; OR = orientation flight; HW = arrival at halfway; AP = approach to the cage containing the lure (within 10 cm); LS = landing on the cage containing the lure.

TABLE 3. ATTRACTION OF *S. exigua* MALE MOTHS TO DIFFERENT LURES IN A CHINESE CABBAGE FIELD[a]

Lure	Total moths captured ±SE/trap[b]
P + phenylacetaldehyde	107.7 ± 7.7a
P + benzaldehyde	84.7 ± 4.6bcd
P + (*Z*)-3-hexenol	96.7 ± 9.5abc
P + (*Z*)-3-hexenyl acetate	99.7 ± 9.0ab
P + (*E*)-2-hexenal	111.0 ± 1.0a
P + soybean oil	76.7 ± 1.9d
P	80.0 ± 5.5cd

[a]The dosage of sex pheromone (P) in lures was 100 μg, and the dosage of each plant volatile was 4000 μg in 10-μl soybean oil.
[b]Means followed by a different letter are significantly different at $P < 0.05$ (Least significant difference test).

phenylacetaldehyde, and P +(*Z*)-3-hexenyl acetate enhanced moth catches by 38.8%, 34.6%, and 24.6%, respectively, compared with traps baited with synthetic sex pheromone alone. Of all the lures tested, however, only the lures with P + benzaldehyde or P +(*Z*)-3-hexenol did not attract significantly more male *S. exigua* than the lures with synthetic sex pheromone alone ($P > 0.05$) (Table 3).

The second trapping experiment in the cotton field, with the exception of (*E*)-2-hexenal, caught more males per day in traps baited with synthetic pheromone plus an individual volatile in a ratio of 1:10 than the number caught in traps baited with synthetic pheromone alone (LSD, $P < 0.05$) (Table 4). However, traps baited

TABLE 4. ATTRACTION OF MALE *S. exigua* MOTHS TO DIFFERENT LURES IN A COTTON FIELD

Lure	Ratio (P/plant volatile)	Mean catch ± SE/ trap/day[a]
P	—	39.0 ± 7.7cd
P + phenylacetaldehyde	1:1	59.5 ± 17.4b
P + phenylacetaldehyde	1:10	56.8 ± 24.8b
P + phenylacetaldehyde	1:100	29.9 ± 7.9cd
P + (*E*)-2-hexenal	1:10	51.0 ± 13.8bc
P +(*Z*)-3-hexenyl acetate	1:10	96.9 ± 34.1a
P + (*Z*)-3-hexenol	1:10	93.2 ± 44.3a
P + benzaldehyde	1:10	87.2 ± 47.4a

[a]Means followed by a different letter are significantly different at $P < 0.05$ (Duncan's multiple range test).

with a combination of synthetic pheromone and phenylacetaldehyde at a 1:100 ratio did not enhance catches compared with traps baited with synthetic pheromone alone.

DISCUSSION

The enhancement of attraction to synthetic semiochemicals by host-plant volatiles has been frequently reported in beetles, but there are only few reports in lepidopterans. Our results show that a combination of sex pheromone and selected plant volatiles enhanced the field captures of beet armyworm males. The blend with pheromone and (*Z*)-3-hexenyl acetate or with phenylacetaldehyde attracted more beet armyworm males than the blend with pheromone alone in cabbage or cotton fields. The results of physiological and behavioral tests in the indoor bioassays were consistent with those from the field tests. This suggests that (*Z*)-3-hexenyl acetate and phenylacetaldehyde show promise in the design of new IPM approaches.

The enhancement effects vary in target species and with the environmental background where the attraction is tested. Reddy and Guerrero (2000) reported that mixtures of (*Z*)-3-hexenyl acetate, (*E*)-2-hexenal, and (*Z*)-3-hexen-1-ol with female sex pheromone induced attractant/arresting behavior in 80–100% of the *Plutella xylostella* males tested, which was significantly higher than the effect induced by the pheromone alone. In addition, (*Z*)-3-hexenyl acetate had a synergistic effect in the capture of *P. xylostella* males in the field. Heptanal enhanced the responses of *Helicoverpa armigera* males to female sex pheromone when it was combined with synthetic pheromone (Fang and Zhang, 2002).

The application of plant volatiles may need to reflect the environment where they will be used, and the fine tuned signal system of each insect. Benzaldehyde enhanced the attractiveness of P in the wind tunnel bioassay (χ^2 test, $P < 0.001$) and in the cotton field experiment (Duncan's test $P < 0.05$) but not in the Chinese cabbage field test (LSD, $P > 0.05$). Although (*Z*)-3-hexenol did not increase the attractiveness of P in the wind tunnel bioassay, it enhanced the capture in the cotton field test (Duncan's test $P < 0.05$). (*E*)-2-Hexenal enhanced male captures in the Chinese cabbage experiment (LSD, $P < 0.05$) but not in the cotton field experiment (Duncan's test $P > 0.05$). In the cotton field experiment, traps baited with a combination of synthetic pheromone and phenylacetaldehyde in a ratio of 1:1 or 1:10 enhanced catches compared with traps baited with synthetic pheromone alone or in a ratio of 1:100. In addition, crop types may also affect the attractiveness of host plant volatile and sex pheromone combinations.

Behavioral mechanisms underlying the enhancement of attraction to sex pheromones have not been well studied. Enhanced male behavioral responses to mixtures of female sex pheromones and plant volatile compounds are due at least in part to enhancement of pheromone-specific pathways (Ochieng et al., 2002). Since

plant volatiles may selectively stimulate pheromone receptor neurons and, thus, affect the quality of perceived pheromone blend, plant odors may be involved in cessation of pheromone-mediated flight (Baker et al., 1988; Dickens et al., 1993b).

Phytophagous insects often meet, court, and mate on host plants, and their sexual behavior may occur principally or exclusively on host plants (Landolt and Phillips, 1997). For a phytophagous species, attraction to host plants may be correlated with attraction to sex pheromones. The scent of a combination of sex pheromones and host plant volatile compounds may suggest the existence of not only the opposite sex, but also a suitable host plant for larvae (Dickens et al., 1993a).

Cues that enhance the attractiveness of sex pheromone lures are of great importance in the development of pheromone-based IPM strategies for insect control. Merely increasing the dosage of sex pheromone on the lures did not significantly enhance catches of *S. exigua* in the field (Wakamura, 1987). Therefore, combinations may be the basis for a more efficient lure for integrated management of *S. exigua*.

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SEX PHEROMONE COMPONENTS OF THE BRONZED CUTWORM, *Nephelodes minians*, A PREY SPECIES OF A BOLAS SPIDER, *Mastophora hutchinsoni*

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Abstract—The bolas spider, *Mastophora hutchinsoni*, attracts adult males of four species of moths by aggressively mimicking their sex pheromone. Here, we report the identification of two sex pheromone components of one of these species, the bronzed cutworm, *Nephelodes minians*. Simultaneous gas chromatographic (flame ionization detection) and electroantennographic detection (EAD) analysis of extracts of the sex pheromone glands of female *N. minians* indicated two components eliciting strong EAD responses from a conspecific male antenna that corresponded in retention time with (*Z*)-11-hexadecenal (Z11-16:Ald) and (*Z*)-11-hexadecenyl acetate (Z11-16:OAc), using both polar and nonpolar columns. Mass spectra of these two peaks were identical to those of synthetic Z11-16:Ald and Z11-16:OAc. The double-bond positions were confirmed by GC–MS analysis of dimethyl disulfide derivatives of the putative pheromone components. In a field test, a 5:1 blend of Z11-16:Ald to Z11-16:OAc attracted male *N. minians*. Overall, there is no overlap in the chemical constituents of the sex pheromones of three moth species preyed upon by this bolas spider for which pheromones have now been identified. These studies suggest that bolas spiders are versatile in their ability to synthesize semiochemicals for different prey species. This versatility may be advantageous to a predator that has an otherwise extremely specialized hunting tactic.

Key Words—Sex pheromone, *Nephelodes minians*, (*Z*)-11-hexadecenal, (*Z*)-11-hexadecenyl acetate, chemical mimicry, bolas spider, *Mastophora hutchinsoni*.

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INTRODUCTION

Bolas spiders attract their male lepidopteran prey by mimicking the sex pheromones emitted by female moths (Eberhard, 1977; Stowe et al., 1987; Yeargan, 1988, 1994; Gemeno et al., 2000). The adult female of the bolas spider *Mastophora hutchinsoni* attracts four species of moths including the smoky tetanolita, *Tetanolita mynesalis* (Walker)(Noctuidae); the bristly cutworm, *Lacinipolia renigera* (Stephens) (Noctuidae); the bronzed cutworm, *Nephelodes minians* Guenée (Noctuidae); and the bluegrass webworm, *Parapediasia teterrella* (Zincken) (Pyralidae) (Yeargan, 1988, 1994). Pheromones have been identified from the two principal prey species, *L. renigera* and *T. mynesalis* (Haynes, 1990; Haynes et al., 1996). The pheromone blend of *L. renigera* is composed of 30:1 blend of (*Z*)-9-tetradecenyl acetate and (*Z*, *E*)-9,12-tetradecadienyl acetate, and that of *T. mynesalis* is a 2:1 blend of (3*Z*,9*Z*)−6*S*,7*R*−epoxy-heneicosadiene and (3*Z*,6*Z*,9*Z*)-heneicosatriene. Whereas the pheromone for *P. teterrella* has not been identified, an optimized attractant consists of a 20:1 blend of (*Z*)-11-hexadecenal and (*Z*)-9-hexadecenal (Clark and Haynes, 1990). For these three species, there is no overlap in the composition of the pheromone or attractants.

Nephelodes minians is a North American noctuid species that is found primarily east of the Rocky Mountains (Covell, 1984; Ayre and Lamb, 1990). The larvae feed mainly on corn and grasses and may cause economic damage to pastures and grassland in some parts of their geographical range (Ayre and Lamb, 1990). This species has one generation per year (Covell, 1984; Ayre and Lamb, 1990). The seasonal flight of adult males occurs during the late summer and early fall at a time when adult female *M. hutchinsoni* hunt using a bolas (a sticky droplet at the end of a short thread) (Yeargan, 1988). In terms of the total number of moths captured by this bolas spider, *N. minians* is a relatively minor prey species (3.7% of total), but it is by far the largest species captured (mean weight is 194.7 mg, compared to 65.5 mg for *L. renigera* and 11.8 mg for *T. mynesalis*) (Yeargan, 1988). The aim of the present study was to identify the sex pheromone components of *N. minians,* in order to gain further insight into the potential biochemical versatility of the bolas spider, *M. hutchinsoni*.

METHODS AND MATERIALS

Chemicals. Synthetic (*Z*)-11-hexadecenyl acetate (Z11-16:OAc) (>99% purity) and (*Z*)-11-hexadecenal (Z11-16:Ald) (>96% purity) were obtained from the Institute of Pesticide Research, Wageningen, the Netherlands (now PheroBank), and Scentry Inc., Buckeye, AZ, respectively. Hydrocarbon standards were purchased from Alltech Associates, Deerfield, IL.

Insects. Adults of bronzed cutworm, *N. minians*, were collected from a blacklight trap set out at a suburban backyard in Richmond, KY, during late September 1995 through 1998. Captured male and female moths were brought to the laboratory, where males were used in gas chromatography/electroantennogram (GC–EAD) studies, and the sex pheromone glands of females were dissected and extracted.

Initially, our goal was to establish a colony from these field-collected moths so that we could evaluate the pheromone blend from known virgin females. However, this proved to be difficult because of the obligate diapause in the egg stage. Another complicating factor was that many of the adult female *N. minians* proved to be previously mated (i.e., spermatophores were found upon dissection). For these reasons, we attempted to stimulate pheromone biosynthesis in these often mated females by injecting them with 2 pmol of pheromone biosynthesis activating neuropeptide (Hez-PBAN, Peninsula Laboratories Inc., Belmont, CA) in 10 μl of buffer solution (0.35 M sucrose, 0.1 M sodium phosphate buffer, pH 6.8) through the intersegmental membrane between the 5th and 6th abdominal segments. After 0.5-h incubation, female pheromone glands were dissected and immediately extracted in hexane containing 50 ng of two internal standards (*n*-pentadecane and *n*-tricosane) for 30 min. The gland was then removed from the solvent. The excess solvent was evaporated under an N_2 stream before injection of the concentrated extract into a gas chromatograph.

Electrophysiological and Chemical Analyses. The gas chromatography–electroantennographic detection system (GC–EAD) used was similar to that described by Struble and Arn (1984) and Leal et al. (1992). A Hewlett-Packard 5890 Series II GC was equipped with either a DB-WAX or a DB-5 column (30 m $\times$ 0.25 mm i.d., J&W Scientific, Folsom, CA). The GC effluent was split at a 1:1 ratio between a flame ionization detector (FID) and an electroantennographic detector (EAD). The injector temperature was held constant at 250°C and the split valve was opened 1 min after injection. The column was held at 80°C for 2 min following the injection and then increased to 230°C at 20°C/min.

An antenna was cut off at its base using fine dissecting scissors, and the terminal segments of the antenna were removed. The excised antenna was placed between two reservoirs of Pringle's saline (Pringle, 1938), which were in contact with silver–silver chloride electrodes connected through a high impedance probe to a Grass P16 amplifier (Quincy, MA). A passive high-pass filter controlled for baseline drift (Struble and Arn, 1984). A Dataq Model DI-420 Signal Conditioning Module (Akron, OH) was used to receive both amplified EAD and FID signals, and the signals were then analyzed by using Dataq MCA-CODAS software (Akron, OH). Six independent gland extracts were evaluated using antennae from six different males.

Mass spectra of GC-EAD active compounds were obtained on a Hewlett-Packard 5890 Series II gas chromatograph equipped with a DB-WAX and a DB-5 column (30 m × 0.25 mm i.d., J&W Scientific, Folsom, CA), linked with an Hewlett-Packard 5972 mass selective detector (MSD, electron impact 70 eV). The GC operating conditions were the same as those described for GC-EAD analysis above. Pooled extracts with two female equivalents from PBAN-treated females were evaluated in initial runs, but these tests were repeated with those containing one female equivalent. The mating status of these females was not determined. The flow rate of helium through the column was kept at 1 ml/min. The scan range of the MSD was set from 40 to 400 amu.

The double-bond positions of pheromone candidates were determined by the procedure of Buser et al. (1983). A female extract (one female equivalent) was mixed with 100 μl of dimethyl disulfide (DMDS) and 10 μl of iodine solution (60 mg of I_2 in 1 ml of diethyl ether) in a 1-ml vial closed with a Teflon-lined cap. The reaction was carried out in an oven at 40°C overnight. Hexane (200 μl) then was added, followed by a 5% solution (100 μl) of $Na_2S_20_3$ in distilled water. The hexane layer was removed and concentrated under N_2 to about 2 μl for GC-MS analysis. Similar procedures were followed for synthetic (*Z*)-11-hexadecenyl acetate (Z11-16:OAc) and (*Z*)-11-hexadecenal (Z11-16: Ald).

Fourteen additional field-captured females were used as part of an experiment to determine the impact of PBAN and mating status on the quantity of Z11-16:Ald and Z11-16:OAc. These females were treated with PBAN in buffer ($N = 7$) as described above, or they were injected with buffer only ($N = 7$). Immediately after the sex pheromone gland was removed and placed in hexane, the female was dissected to determine presence or absence of a spermatophore in the bursa copulatrix. Two-way (PBAN and mating status) analyses of variance were conducted for both compounds (Anonymous, 2003).

Field Tests. Field tests were conducted from October 4 to 7, 1995, and from September 16 to 24, 1996, at the University of Kentucky's South Farm, Lexington, KY, and a suburban backyard in Richmond, KY. Synthetic blends were prepared in hexane. Red rubber septa which had been previously extracted twice in hexane (A. H. Thomas, Swedesboro, NJ) were used as dispensers. Pherocon™ 1C traps were hung from PVC or steel posts so that the trap opening was approximately 0.5 m above the ground. Within a replicate, each trap was set at least 10 m apart. The traps were checked daily, and their positions within a replicate were randomized to minimize the effects of habitat heterogeneities. If no moths were captured within a block, then that block was not used in a Kruskal–Wallis nonparametric analysis of variance ($N = 32$, 8 out of 40 initial replicates discarded because of this criterion; Anonymous, 2003).

RESULTS AND DISCUSSION

Electrophysiological and Chemical Analyses. Analysis of female extracts using GC-EAD showed that two compounds elicited strong and consistent responses from conspecific male antennae (Figure 1). No other consistent responses

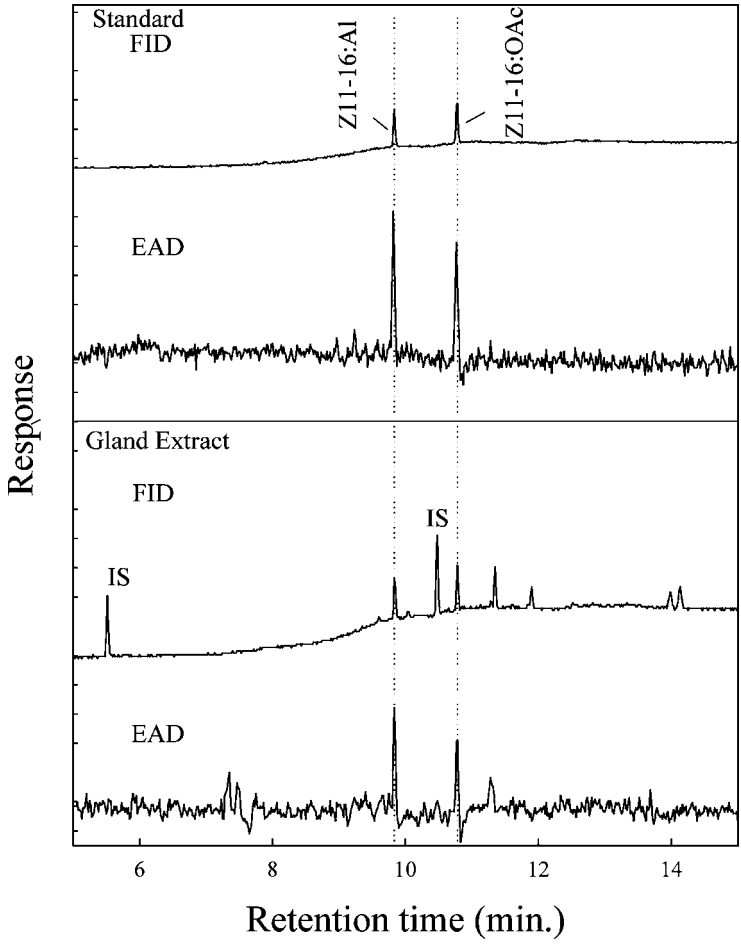

FIG. 1. GC–EAD analyses using a DB-Wax column of a standard solution containing Z11-16:Al and Z11-16:OAc (upper two traces) and an extract of the sex pheromone gland of the bronzed cutworm moth (lower two traces). Only EAD responses at the retention times of Z11-16:Ald and Z11-16:OAc were consistently found to gland extracts. Internal standards (IS) were 15:Hc and 23:Hc.

were noted. The retention times for these EAD-active peaks matched those of Z11-16:Ald and Z11-16:OAc by using both the DB-Wax and the DB-5 columns. The mass spectrum of the first EAD active peak showed diagnostic ions at *m/z* 55 and 220 (M-18), and the molecular ion at *m/z* 238 characteristic of a monounsaturated C_{16} aldehyde. The mass spectrum with the characteristic ions of *m/z* 61 and *m/z* 222 (M-60) found from the second compound indicated a monounsaturated C_{16} acetate.

The determination of the double-bond position of this aldehyde and acetate solely by their retention times can be uncertain. The dimethyl disulfide derivatives of a pheromone extract yielded products with retention times and mass spectra that matched those of dimethyl disulfide derivatives of Z11-16:Ald and Z11-16:OAc, respectively. The key ions for the derivative of Z11-16:Ald were *m/z* 117, 215, and 332. The key fragment ions at *m/z* 117, 199, 259, and 376 matched those of the derivative of authentic Z11-16:Ac. This evidence confirmed the double-bond position at the 11 position for both compounds. The retention times of E11- and Z11-16:Ald differ by 0.04 min under these operating conditions using a DB-Wax column with 89% return to baseline after E11-16:Ald elutes (test run with about 20 ng of each isomer). The retention times of E11-16:OAc and Z11-16:OAc differ by 0.05 min with 97% return to baseline after E11-16:OAc elutes (10 ng of each). Because *Z* and *E* isomers can be resolved, the identification of the two EAD active peaks as Z11-16:Ald and Z11-16:OAc was supported. However, at this level of resolution, we cannot preclude the possibility that there could be a trace amount of the *E*-isomers in the pheromone blend (i.e., a trace amount of an *E*-isomer might not be apparent in the leading edge of a *Z*-isomer).

The quantity and blend ratio of Z11-16:Ald and Z11-16:OAc were extremely variable in individual gland extracts ($N = 14$). Z11-16:Ald ranged in abundance from 0.1 to 12.8 ng, whereas Z11-16:OAc ranged from 0.1 to 121.5 ng. Treatment with PBAN stimulated pheromone biosynthesis [PBAN-treated: Z11-16:Ald = 6.5 ng $\pm$ 1.3* and Z11-16:OAc = 30.5 $\pm$ 16.2 ($N = 7$) compared to buffer-treated: Z11-16:Ald = 2.5 ng $\pm$ 0.5 and Z11-16:OAc = 9.9 $\pm$ 4.5 ($N = 7$), asterisk indicates a significant difference from the control group; two-way analysis of variance; $P < 0.05$]. The mating status of the females also influenced the quantity of these two compounds recovered [virgin Z11-16:Ald = 6.5 ng $\pm$ 1.8 and Z11-16:OAc = 45.0 $\pm$ 19.3* ($N = 5$), compared to mated: Z11-16:Ald = 3.3 ng $\pm$ 0.8 and Z11-16:OAc = 6.5 $\pm$ 4.0 ($N = 9$); asterisk indicates a significant difference between mated and virgin; two-way analysis of variance; $P < 0.05$]. The mean percent of Z11-16:Ald in the two-component blend ranged from 3.6 to 93.9% (calculated as 100 times the quantity of Z11-16:Ald divided by the sum of the quantities of Z11-16:Ald and Z11-16:OAc), The extremely high variance in pheromone blend ratio may be attributed to mating status, PBAN-treatment, unknown age of females, normal periodicity of pheromone production, and impact of the light trap on the moths. In addition, it is possible that there may be distinctions

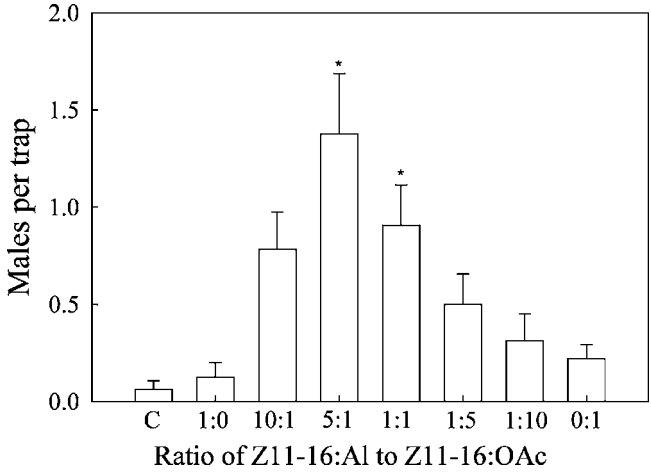

FIG. 2. Mean number of male bronzed cutworms caught per trap per night with different blend ratios of Z11-16:Al and Z11-16:OAc ($N = 32$). There was 100 μg of the most abundant component of the blend. Asterisk indicates means that were significantly different than the control treatment.

between how the aldehyde and acetate components are synthesized, stored, and/or released.

Field Tests. A 5:1 blend of Z11-16:Ald to Z11-16:OAc was most effective in capturing males (Figure 2). Traps baited with 5:1 and 1:1 blends caught more males than control traps ($P < 0.05$; Kruskal–Wallis nonparametric analysis of variance with pairwise comparisons of treatments and controls). Few male bronzed cutworms were attracted to either pure Z11-16:OAc or pure Z11-16:Ald. Thirty-two individuals of a single crambid species were caught with Z11-16:Ald, but none were caught with any of the blends of Z11-16:Ald and Z11-16:OAc. No other species of commonly caught male moths were noted. The number of male bronzed cutworms caught with the optimal blend of these two components was low, perhaps indicating missing pheromone components, or low population densities at trapping sites. Because we were unable to rear females in the laboratory, we cannot establish whether or not the optimal blend of these two components would be competitive with virgin females.

Although pheromones have been identified for many species of Noctuidae, this is the first report from the genus *Nephelodes* (Witzgall et al., 2004). A synthetic mixture of Z11-16:Ald and Z11-16:OAc in a 1:1 ratio was reported as a sex attractant for male glassy cutworms, *Crymodes devastator*, and this mixture also captured small numbers of male bronzed cutworm moths (Steck et al., 1977; Underhill et al., 1977). The results of our experiments suggest that synthetic

PBAN may be useful for the identification of pheromone products from moths of unknown mating status, such as would be retrieved from blacklight traps, as was the case in our study. Tabata et al. (2003) used a similar approach to quantify pheromone polymorphism within field populations of *Ostrinia scapulalis* in Japan. By injecting the synthetic C-terminal octapeptide of PBAN into field-collected females, they were able to determine the pheromone phenotype of females that had already mated. Whereas we were able to determine two pheromone components for *N. minians* by injecting mated females with PBAN, we found extreme variability of pheromone blend ratios. Some of the variation could be due to the fact that *N. minians,* unlike *Ostrinia*, use both an aldehyde and an acetate pheromone component. More methodical experiments, controlling mating status, age, and time of day might provide greater precision in determination of the pheromone blend ratio. However, control of these variables may not always be possible for insects with obligate diapause (i.e., difficult to obtain virgin adult female moths).

The results of this study indicate that the sex pheromone of *N. minians* does not share chemical components with two other prey species of the bolas spider *M. hutchinsoni*. The sex pheromone of the most frequently captured prey species (*T. mynesalis*) consists of 2:1 blend of (3*Z*,6*Z*)-6*S*,7*R*-epoxyheneicosadiene and (3*Z*,6*Z*,9*Z*)-heneicosatriene (Haynes et al., 1996), whereas the sex pheromone for *L. renigera* consists of (*Z*)-9-tetradecenyl acetate and 3% (*Z*,*E*)-9,12-tetradecadienyl acetate. An effective sex attractant for *P. teterrella*, another prey species of this bolas spider, consists of a 20:1 blend of Z11-16:Ald and Z9-16:Ald. Thus, it seems likely that there will be overlap in the chemical composition of the pheromone of *P. teterrella* and *N. minians*. Interestingly, Z11-16:Ald by itself was not an effective attractant for either *P. teterrella* (Clark and Haynes, 1990) or *N. minians* (this study).

This study, considered with earlier reports, provides indirect evidence that bolas spiders are capable of producing a diverse set of allomones (see Stowe et al., 1987; Yeargan, 1994; Haynes et al., 1996, 2002) that mimic acetate, aldehyde, hydrocarbon, and epoxide pheromone components of different chain lengths. To date only Z9-14:OAc and Z,E-9,12-14:OAc, components of *L. renigeria* pheromone, have been detected in the effluvia of *M. hutchinsoni* (Gemeno et al., 2000). These were detected by EAD, with the quantities emitted often below the limits of detection of FID. Detection of pheromone components of the other species from bolas spiders may also require the use of the antennae of the moth prey as detectors. Haynes et al. (2002) found that the bolas spider reduces the impact of behavioral antagonism between pheromone blends of *L. renigera* and *T. mynesalis* by emitting less of the 14 carbon acetates late at night when male *T. mynesalis*, the species that uses the hydrocarbon and epoxide pheromone, are active. However, *N. minians*, *T. mynesalis*, and *P. teterrella* are all caught by this bolas spider after 10:30 P.M. Whereas *L. renigera* and *T. mynesalis* are the most common prey captured (together over 90% of individuals captured), it remains to be determined if the

bolas spider is efficient (percent attracted out of number available) in attracting all four species.

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CHEMICAL, ELECTROPHYSIOLOGICAL, AND BEHAVIORAL INVESTIGATIONS ON THE SEX PHEROMONE OF LACKEY MOTH, *Malacosoma neustrium*

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Abstract—The lackey moth *Malacosoma neustrium* (L.) (Lepidoptera Lasiocampidae) is a common pest of many forest and cultivated broadleaf trees. Analysis by GC-EAD of gland extracts and female effluvia showed the presence of two active peaks that were characterized as (*E*,*Z*)-5,7-dodecadienal (*E*5,*Z*7-12:Ald) and (*E*,*Z*)-5,7-dodecadienol (*E*5,*Z*7-12:OH) according to their GC retention times, mass spectra, and electroantennographic activity. (*E*,*Z*)-5,7-dodecadienyl acetate (*E*5,*Z*7-12:Ac) was also detected in the gland extracts. The average amounts of *E*5,*Z*7-12:Ald, *E*5,*Z*7-12:OH, and *E*5,*Z*7-12:Ac extracted from an abdominal tip were 113.2 ± 22.0 ng, 29.9 ± 6.2 ng, and 11.6 ± 2.0 ng, respectively. In the effluvia from single females, on the average, 1.9 ± 0.7 ng/min of aldehyde and 0.3 ± 0.1 ng/min of alcohol were collected. In wind tunnel tests, male behavioral sequences elicited by *E*5,*Z*7-12:Ald alone or in 3:1 and 9:1 blends with the corresponding alcohol were similar to those evoked by one female equivalent of sex pheromone gland extract. Field trapping experiments showed that *E*5,*Z*7-12:Ald is essential to attract male moths. On adding *E*5,*Z*7-12:OH to *E*5,*Z*7-12:Ald in 1:3 and 1:9 ratios, male attraction slightly increased whereas in a 1:1 ratio, the attractiveness of *E*5,*Z*7-12:Ald was reduced.

Key Words—*Malacosoma neustrium*, sex pheromone, (*E*,*Z*)-5,7-dodecadienal, (*E*,*Z*)-5,7-dodecadienol, electroantennography, wind tunnel, field trapping.

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INTRODUCTION

The lackey moth *Malacosoma neustrium* (L.) (Lepidoptera Lasiocampidae) is a univoltine pest present in Europe and Asia. Larvae feed on many broadleaf trees, especially *Quercus* spp. and occasionally on cultivated plants. In Italy, recent outbreaks in Sardinia and Calabria have caused heavy defoliations of cork oak (*Quercus suber* L.) and holm oak (*Quercus ilex* L.) forests (Luciano and Roversi, 2001).

A pheromone-based monitoring system could be a useful tool for improving control strategies for this species. The sex pheromone of *M. neustrium* was first reported incorrectly as a mixture of (*Z*)-5-decenyl acetate (*Z*5-10:Ac) and (*Z*)-5-dodecenol (*Z*5-12:OH) (Melikyan et al., 1980). In field trails carried out in Sardinia, these compounds did not attract *M. neustrium* male moths (Luciano et al., 1982).

Investigations on a Russian strain by gas chromatography (GC), gas chromatography coupled to mass spectrometry (GC-MS), and electroantennography (EAG) showed the presence of (*E*,*Z*)-5,7-dodecadienal (*E*5,*Z*7-12:Ald), (*Z*,*E*)-5,7-dodecadienal (*Z*5,*E*7-12:Ald), (*E*,*Z*)-5,7-dodecadienol (*E*5,*Z*7-12:OH), (*Z*,*E*)-5,7-dodecadienol (*Z*5,*E*7-12:OH), (*E*,*Z*)-5,7-dodecadienyl acetate (*E*5,*Z*7-12:Ac), and (*Z*,*E*)-5,7-dodecadienyl acetate (*Z*5,*E*7-12:Ac) in a ratio of 1.6: 0.3: 1.0: 0.1: 0.6: 0.8 in solvent extracts from female abdominal tips (Konyukhov and Kovalev, 1988). In preliminary wind tunnel and field tests, *E*5,*Z*7-12:Ald or a 3:1 blend of *E*5,*Z*7-12:Ald and *E*5,*Z*7-12:OH were reported to be attractive to male moths but the authors reported no statistical analyses, or details about doses, dispensers, and traps used. They also emphasized the need for further laboratory and field studies (Konyukhov and Kovalev, 1988).

In order to contribute to the development of a *M. neustrium* monitoring system, the goal of the present work was to determine whether the sex pheromone components reported for the Russian strain were also present in the Italian one, and to fully evaluate the biological activity of the identified compounds. We examined the compounds present in solvent extracts and effluvia from female sex pheromone glands of *M. neustrium*, the sensory responses of male moths to both the female-produced and synthetic compounds, and male behavioral responses both in a laboratory wind tunnel and in field bioassays.

METHODS AND MATERIALS

Insects. Cocoons were collected from an infested *Q. suber* forest in Sardinia (Italy) during 2001–2002 at the end of May. Pupae were sexed and kept in groups

($N = 20$) in filter paper-lined plastic containers (30 × 20 × 8 cm) at 28 ± 2°C, 65 ± 5% R.H. and L16:D8 photoperiod until emergence. Adults were separated daily and isolated in transparent plastic containers (6 cm i.d. × 8 cm) covered with a fine mesh net (1 mm) and supplied with 10% sucrose solution soaked on cotton wicks.

Female Calling. The diel rhythm of calling behavior was observed with 1- to 3-d-old virgin females ($N = 30$) individually placed in plastic containers and held under the environmental conditions described above. Observations were made under red light (3 lux) at 1hr intervals during the scotophase.

Gland Extracts. An abdominal tip, including the pheromone gland, was excised from a female calling for 30 min during the first scotophase and immersed in hexane (20 μl) for 10 min at room temperature. Extracts from 50 abdominal tips were combined and concentrated to 1 female equivalent/μl (FE/μl) using a slow stream of nitrogen, and stored at −20°C until needed. For pheromone titer determination, additional extracts ($N = 10$) were prepared from individual abdominal tips and 100 ng of (*E*)-7-tetradecenyl acetate (*E*7-14:Ac) was added as an internal standard.

Female Effluvia. Airborne volatiles were collected from single 1-d-old virgin females ($N = 20$) calling for 30 min during the first scotophase. The female was placed in an appropriately shaped pipette tip that allowed for the extrusion of the last urites. This was suspended, by a polyurethane foam (PUF) support (cleaned with hexane for 12 hr in a Soxhlet apparatus) in a conical glass container (6 ml) into which dried (silica gel) and purified (activated charcoal) synthetic air flowed (70 ml/min) for 15 min at 28°C. Effluvia were adsorbed onto the surface of two glass capillaries (1 mm i.d.; 200 mm long) mounted in series at the outlet of the glass container (1 mm i.d.) and kept at about 2–4°C with chilled aluminium blocks. The capillaries and the glass container were connected by Teflon tube sleeves with the glass surfaces in contact. Each capillary was rinsed with redistilled hexane (2 μl ×2) containing 25 ng of *E*7-14:Ac as an internal standard. The hexane rinse (1.5–2 μl) was collected in a 5 μl syringe (Hamilton, Switzerland) and directly injected into the gas chromatograph (GC) for qualitative and quantitative analyses. Before the extraction, the glass container was rinsed with hexane and dried (250°C for 12 hr). After each collection, the Teflon tube, pipette tip, and PUF support were replaced (Rotundo et al., 2001).

Chemicals. *E*7-14:Ac (purity ≥ 98%) used as internal standard was purchased from the Institute for Plant Protection, Wageningen, The Netherlands. *E*5,*Z*7-12:Ald, *E*5,*Z*7-12:OH, *E*5,*Z*7-12:Ac, *Z*5,*E*7-12:Ald, *Z*5,*E*7-12:OH, and *Z*5,*E*7-12:Ac (purity ≥ 91.5%) were supplied by Novapher (San Donato Milanese, Milano, Italy).

*Gas chromatography–Electroantennographic Detection (*GC-EAD*).* Gland extracts and female effluvia were analyzed by GC-EAD using a Fisons 9000 series

GC equipped with a splitless injection system and a SPB-5 column (30 m × 0.32 mm i.d., 0.25 μm film thickness, Supelco Inc., Bellefonte, PA). Conditions were: carrier gas, helium at 20 psi; oven program, 60°C for 2 min, 10°C/min to 250°C; injector and detector temperature, 250°C. The column effluent was split equally by an "X" connector (Supelco Inc.) between a flame ionization detector (FID) and an EAG male moth antennal preparation (see below) with 15 ml/min helium supplied through the extra arm of the X-cross. The EAD responses were amplified (50x) with an AC/DC UN-6 amplifier in DC mode (Syntech Laboratories, Hilversum, The Netherlands).

Gas chromatography-Mass Spectrometry (GC-MS). Analyses were performed with a Fisons 8000 series GC linked to a MD800 (Fisons) quadrupole mass detector. Capillary column, carrier gas, oven program, and injection conditions were the same as used for GC-EAD. Mass spectra were obtained at 70 eV with the ion source at 200°C. GC-EAD active compounds were tentatively identified by observing characteristic ions and comparing their spectra with those of authentic compounds.

In order to confirm the identity of GC-EAD active peaks, their retention times were compared with those of authentic compounds by using a SPB-5 column (30 m × 0.32 mm i.d.) and a CP-Wax CB52 column (30 m × 0.25 mm i.d., 0.25 μm film thickness, Chrompack, The Netherlands; 70°C for 2 min, to 210°C at 10°C/min; injector and detector temperature, 220°C). Candidate sex pheromone components in the extracts from individual abdominal tips and female effluvia were quantified using the SPB-5 column, by comparison of the peak areas to that of *Z*7-14:Ac.

Electroantennography. The EAG technique was similar to that used in previous studies (Rotundo et al., 1984; Den Otter et al., 1996; De Cristofaro et al., 2000). An antenna was excised at the base from a 1-d-old male and after removing the two distal segments it was mounted between glass electrodes filled with Kaissling's saline (Kaissling and Thorson, 1980). Electrical contact of the antennal preparation with the amplifier was made with AgCl-coated silver wires. Stimuli were serial hexane solutions (0.0001–10 μg/μl) of compounds identified from female sex pheromone glands and some related isomers. Test solutions (10 μl) were applied to filter paper strips (1 cm^2, Whatman No. 1) placed in Pasteur pipettes (15 cm long). Stimuli were puffed into a constant air stream (50 cc/sec) flowing through a stainless steel tube (i.d. 8 mm) and passing continuously over the antenna. During 0.1 sec, 2.5 cc of vapor from an odor cartridge were added. Intervals between stimuli were 30 sec. Before and after stimulation with the same dose of three randomly selected test compounds, a reference stimulus (100 ng of E5,*Z*7-12:Ald) was applied to check the constancy of the antennal responsiveness. Each stimulus was tested on antennae of 10 different males. For each dose of test compounds, EAG responses were submitted to analysis of variance (ANOVA), and means were separated with the Tukey multiple range test ($P < 0.05$).

Wind-Tunnel Bioassays. A glass wind tunnel (250 × 60 × 60 cm) was used to test the biological activity of 12-carbon diunsaturated *E*,*Z* and *Z*,*E* aldehydes, alcohols and acetates, and binary blends (1:1, 3:1, 9:1) of *E*5,*Z*7-12:Ald and *E*5,*Z*7-12:OH to *M. neustrium* males. The activity of different lures was compared with that of 1 FE of gland extract.

Tests were carried out under red light (3 lux) at 23 ± 2°C, 50 ± 5% RH and an air speed of 0.3 m/sec. The air flow was generated and purified (activated charcoal) by an air speed and humidity-conditioning unit (De Cristofaro et al., 2003) and conducted through two metallic net (3 mm mesh) screens to achieve a laminar flow.

Before each experiment, 10 μl of hexane solution (10 ng/μl) of lure to be tested were distributed on a circular piece of filter paper (1.0 cm^2). After solvent evaporation (3 min), the lure was suspended by a stainless steel holder at a distance of 25 cm from the upwind end of the tunnel in the middle of the cross section.

Experiments were carried out with newly emerged males between the 2nd and the 5th hr of the first scotophase. Males were individually released from a cylindrical (4 cm i.d. × 6 cm) metallic net (3 mm mesh) cage placed 150 cm downwind from the source on a stainless steel jack. Insects were allowed to respond for 30 min. The behavioral sequences recorded were: wing fanning (WF), taking flight (TF), orientation toward odor source (OR), and source contact (SC). Males (20 per treatment) were used once. For each behavioral category, the percentages of responding males were subjected to ANOVA followed by a Ryan's multiple comparison test on proportions (Ryan, 1960) ($P < 0.05$).

Field Test. Field trapping experiments were carried out at Savelli (Calabria Region) during June 13–30, 2003, where the population of *M. neustrium* was estimated to be low. Delta traps (13 × 11 × 22 cm; Novapher, Italy) were baited with polyethylene vials (0.35 ml) containing 0.25 or 1 mg of synthetic *E*5,*Z*7-12:Ald alone or in combination (1:1, 3:1, 9:1) with *E*5,*Z*7-12:OH. Traps were suspended from trees 2–3 m above ground and spaced 50 m apart. Treatments were replicated three times in a randomized block design. At 4-d intervals trap bottoms were replaced and male catches recorded. Data were transformed to $\sqrt{x}+0.5$ and subjected to ANOVA followed by a Least Significant Difference test (LSD) ($P < 0.05$).

RESULTS

Diel Rhythm of Female Calling. Females exhibited calling behavior (extrusion of the last urites) during the first (93%) and the second (82%) scotophases following emergence. Only a few females (13%) survived to the third scotophase, but they did not call.

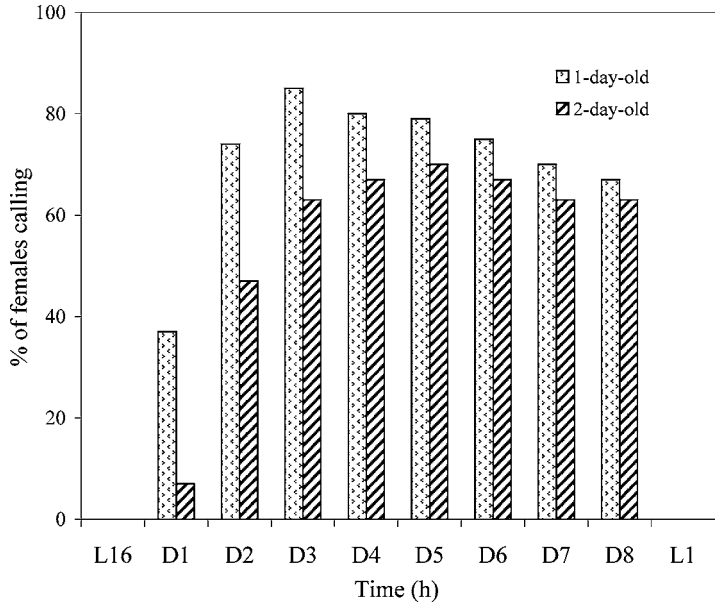

FIG. 1. Diel rhythm of calling behavior of *Malacosoma neustrium* (L.) virgin females.

In the first and second scotophases females started calling during the first hour of darkness, and calling continued until the beginning of the photophase (D1) (Figure 1).

Chemical Analyses. GC-EAD analysis, by a SPB-5 capillary column, of gland extract (1 FE) and female effluvia detected two active peaks at the retention times of 14.07 min (A) and 15.08 min (B) (Figure 2). The mass spectrum of compound A showed diagnostic fragment ions at *m/z* 180 (M^+), 162 (M^+-H_2O), 151 (M^+-CHO), 79 ($C_6H_7^+$), 44 ($CH_2{=}CHO^+$), and 41 ($C_3H_5^+$). The spectrum of compound B presented characteristic fragment ions at *m/z* 182 (M^+), 164 (M^+-H_2O), 153, 79 ($C_6H_7^+$), 41 ($C_3H_5^+$), and 31 (CH_2OH^+). In addition, in the gland extract, GC-MS analysis detected a third compound (C) (Figure 2) with diagnostic fragment ions at *m/z* 224 (M^+), 164 (M^+-CH_3COOH), 79 ($C_6H_7^+$), 61 ($CH_3COOH_2^+$), 43 (CH_3CO^+), and 41 ($C_3H_5^+$). This information suggested that compounds A, B, and C were 12-carbon diunsaturated aldehyde, alcohol, and acetate, respectively. Comparative GC-MS showed that the fragmentation patterns of compounds A, B, and C matched those of synthetic E5,Z7-12:Ald, E5,Z7-12:OH, and E5,Z7-12:Ac, respectively the compounds reported previously from the Russian population (Konyukhov and Kovalev, 1988). Comparison of retention times of the compounds (A, B, C) on polar and low polarity GC columns with those of authentic samples confirmed their identity (Table 1).

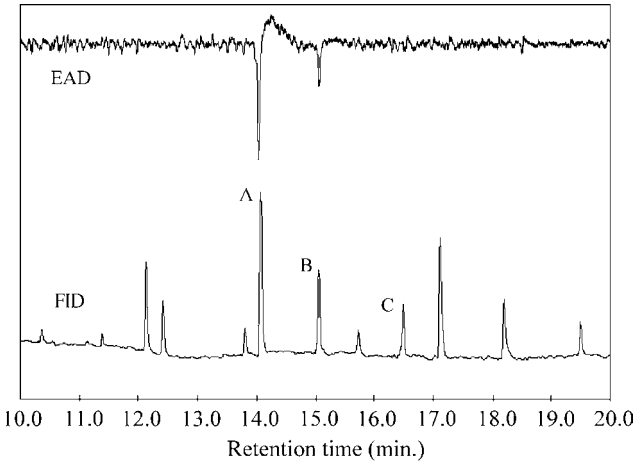

FIG. 2. Simultaneously recorded flame ionization detector (FID) and electroantennographic detector (EAD: male *M. neustrium* antenna) responses to one female equivalent of pheromone gland extract, chromatographed on a SPB-5 capillary column (30 m × 0.32 mm i.d.; oven program: 2 min at 60 °C, 10 °C/min to 250 °C). Identifications of peaks: (A)*E*5,*Z*7-12:Ald; (B)*E*5,*Z*7-12:OH; and (C)*E*5,*Z*7-12:Ac.

Titer of Sex Pheromone Components. From an abdominal tip, on the average, 113.2 ± 22.0 ng of *E*5,*Z*7-12:Ald, 29.9 ± 6.2 ng of *E*5,*Z*7-12:OH, and 11.6 ± 2.0 ng of *E*5,*Z*7-12:Ac corresponding to a 7.3: 1.9: 0.8 ratio were extracted. In the effluvia from single females, on the average, 1.9 ± 0.7 ng/min of *E*5,*Z*7-12:Ald and 0.3 ± 0.1 ng/min of *E*5,*Z*7-12:OH, corresponding to a 9:1 ratio, were found. These compounds were collected only in the first capillary, indicating no breakthrough during the collection period.

EAG. The male EAG responses to different doses of the identified compounds and the *Z*5,*E*7 isomers are reported in Figure 3. The EAGs to *E*5,*Z*7-12:Ald ranged from 0.45 mV at 0.0001 μg to 3.90 mV at 100 μg and, for different doses tested, were significantly ($P < 0.05$) higher than those to the other compounds. *Z*5,*E*7-12:Ald and *E*5,*Z*7-12:OH elicited similar EAGs that increased from 0.37 mV and 0.17 mV at 0.01 μg to 2.25 mV and 1.92 mV at 10 μg, respectively. Other compounds tested did not evoke consistent dose-dependent EAGs.

Wind-Tunnel Bioassays. Using different doses (10 ng to 100 μg) of *E*5,*Z*7-12:Ald, preliminary tests showed that the 100 ng dose allowed for the best male behavioral response under our experimental conditions. In single component tests, only *E*5,*Z*7-12:Ald elicited OR (80%) and SC (75%), which were not different from those induced by 1 FE gland extract (Table 2). Adding *E*5,*Z*7-12:OH to *E*5,*Z*7-12:Ald in a 1:9 or 1:3 ratio, 1 did not increase the response whereas a 1:1

TABLE 1. COMPARISON OF RETENTION TIMES (MIN) OF COMPOUNDS DETECTED IN SEX PHEROMONE GLAND EXTRACTS BY GC-EAD (A, B) AND/OR GC-MS (A, B, C) WITH THOSE OF 12-CARBON DIUNSATURATED *E*5,*Z*7 AND *Z*5,*E*7 ALDEHYDES, ALCOHOLS, AND ACETATES ON POLAR (CP-WAX 52 CB: 70°C FOR 2 MIN, 10°C/MIN TO 210°C) AND LOW POLARITY (SPB-5: 60°C FOR 2 MIN, 10°C/MIN TO 250°C) CAPILLARY COLUMNS

	Compound			Authentic chemical					
Column	A	B	C	*E*,*Z*:Ald	*Z*,*E*:Ald	*E*,*Z*:OH	*Z*,*E*:OH	*E*,*Z*:Ac	*Z*,*E*:Ac
CP-Wax	12.98	15.83	14.96	12.98	13.24	15.82	15.73	14.95	15.27
SPB-5	14.07	15.08	16.59	14.07	14.34	15.08	14.96	16.58	16.96

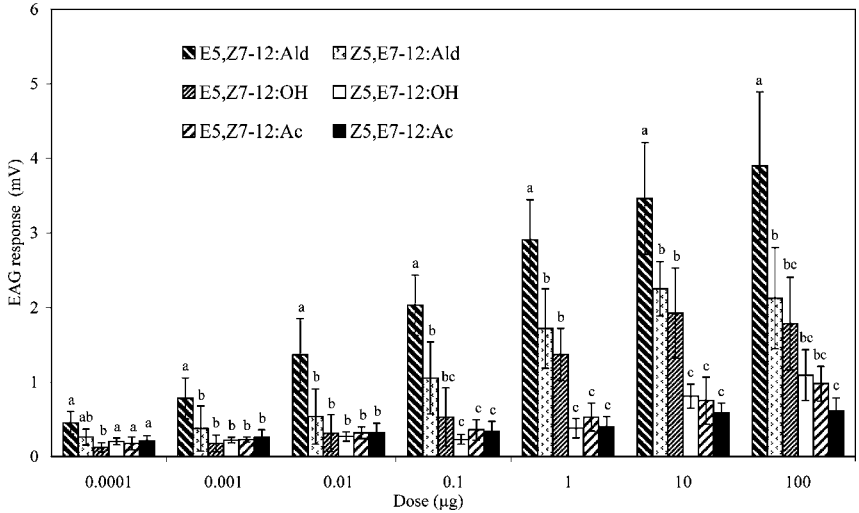

FIG. 3. Mean EAG responses (±SD) of *M. neustrium* males ($N = 10$) to the compounds identified in the sex pheromone gland and some related isomers. Bars in the same dose group with the same letter are not significantly different for $P < 0.05$ (Tukey multiple range test).

ratio of *E*5,*Z*7-12:Ald to *E*5,*Z*7-12:OH resulted in significant reductions to the behavioral sequences (Table 2).

Field. During field trails, 167 males were caught from June 13 to 21. All the lures tested captured *M. neustrium* (Table 3). Male captures by *E*5,*Z*7-12:Ald and the 3:1 and 9:1 blends of *E*5,*Z*7-12:Ald and *E*5,*Z*7-12:OH were similar and significantly higher than those with 1:1 mixture. No significant effects of dose were observed (Table 3).

DISCUSSION

M. neustrium virgin females started calling at the beginning of the first scotophase, suggesting that females are sexually mature at emergence, and calling continued throughout the scotophase.

Two GC-EAD active compounds were found in gland extracts and effluvia from virgin females. The major component was characterized as *E*5,*Z*7-12:Ald and the minor one as *E*5,*Z*7-12:OH according to their retention times and mass spectra. The two compounds are released by virgin females in ratio of 9:1. *E*5,*Z*7-12:Ac was also detected in the gland extracts, but it proved to be GC-EAD inactive. This acetate may be a precursor of other pheromone components. EAG tests

TABLE 2. BEHAVIORAL RESPONSE OF *M. neustrium* MALES TO SYNTHETIC COMPOUNDS, TWO-COMPONENT BLENDS, AND FEMALE GLAND EXTRACT

Composition of the lure (ng)						Behavioral response (%)[a]			
E,*Z*:Ald	*Z*,*E*:Ald	*E*,*Z*:OH	*Z*,*E*:OH	*E*,*Z*:Ac	*Z*,*E*:Ac	WF	TF	OR	SC
100						100 a	100 a	80 a	75 a
	100					65 b	60 b	0 c	0 b
		100				50 b	50 b	0 c	0 b
			100			55 b	50 b	0 c	0 b
				100		60 b	60 b	0 c	0 b
					100	55 b	50 b	0 c	0 b
50		50				55 b	55 b	25 b	0 b
75		25				100 a	100 a	80 a	75 a
90		10				100 a	100 a	90 a	80 a
Female gland extract (1 FE)						100 a	100 a	90 a	85 a

[a] WF: wing fanning; TF: taking flight; OR: orientation; SC: source contact. $N = 20$. Values in the same column followed by the same letter are not significantly different at $P < 0.05$ by Ryan's multiple comparison test on proportions (Ryan, 1960).

TABLE 3. FIELD TRAPPING OF LACKEY MOTH IN SAVELLI, CALABRIA, ITALY (JUNE 13–JUNE 21, 2003)

Attractants (μg)		
*E*5,*Z*7:Ald	*E*5,*Z*7:OH	Total catch per trap[a] (mean ± SD)
1000	—	7.0 ± 1.0 a
900	100	9.7 ± 2.1 a
750	250	9.7 ± 2.5 a
500	500	2.3 ± 2.1 b
250	—	7.7 ± 2.1 a
225	25	9.3 ± 2.1 a
188	62	8.0 ± 1.0 a
125	125	2.0 ± 1.7 b
Blank trap		0.0

[a] Three replicates. Mean followed by the same letter are not significantly different at $P < 0.05$ by LSD multiple range test following ANOVA of $\sqrt{x} + 0.5$ transformed data; control omitted from ANOVA to avoid heterogeneity among the variances (Levene test).

support the results of chemical analyses. The highest responses from antennae of *M. neustrium* males were elicited by *E*5,*Z*7-12:Ald and *E*5,*Z*7-12:OH, whereas acetates elicited minimal EAG responses.

Wind tunnel and field tests indicated that *E*5,*Z*7-12:Ald is essential to attract males. On adding the corresponding alcohol to *E*5,*Z*7-12:Ald in 1:9 ratio, male attraction appeared to increase, whereas in a 1:1 ratio the attractiveness of *E*5,*Z*7-12:Ald was reduced. In the correct ratio, *E*5,*Z*7-12:OH may have potential for enhancing specificity of the communication signal and/or inhibiting the attraction of sympatric species (Linn and Roelofs, 1995).

Z,*E* aldehyde, alcohol, and acetate isomers, previously reported (Konyukhov and Kovalev, 1988) in pheromone gland extracts of the *M. neustrium* Russian strain were not found in the Italian one. This divergence may be due to geographic variation rather than to differences in the extraction techniques. In both studies, solvent extraction was performed on abdominal tips of calling newly emerged females; in addition, the relatively high quantities of compounds extracted in our study suggest that other possible components would have been detected if they had been present. The relevance of the possible minor components to both the Russian and Italian population remains unclear. Careful field trials with different ratios of the isomers will be required before any conclusions can be reached.

*E*5,*Z*7–12:Ald and *E*5,*Z*7–12:OH also have been reported as sex pheromone components of *M. americanum* (Hill and Roelofs, 1980; Kochansky et al., 1996) and *M. californicum* (Underhill et al., 1980) which range across North America.

At the present time in Italy, lackey moth flight activity can be monitored using delta traps and polyethylene dispensers baited with 250 μg of a 9:1 blend of $E5,Z7$-12:Ald and $E5,Z7$:12:OH.

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DISCREPANCY BETWEEN ANTENNAL AND BEHAVIORAL RESPONSES FOR ENANTIOMERS OF α-PINENE: ELECTROPHYSIOLOGY AND BEHAVIOR OF *Helicoverpa armigera* (LEPIDOPTERA)

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Abstract—The ability of adult cotton bollworm, *Helicoverpa armigera* (Hübner), to distinguish and respond to enantiomers of α-pinene was investigated with electrophysiological and behavioral methods. Electroantennogram recordings using mixtures of the enantiomers at saturating dose levels, and single unit electrophysiology, indicated that the two forms were detected by the same receptor neurons. The relative size of the electroantennogram response was higher for the (−) compared to the (+) form, indicating greater affinity for the (−) form at the level of the dendrites. Behavioral assays investigated the ability of moths to discriminate between, and respond to the (+) and (−) forms of α-pinene. Moths with no odor conditioning showed an innate preference for (+)-α-pinene. This preference displayed by naïve moths was not significantly different from the preferences of moths conditioned on (+)-α-pinene. However, we found a significant difference in preference between moths conditioned on the (−) enantiomer compared to naïve moths and moths conditioned on (+)-α-pinene, showing that learning plays an important role in the behavioral response. Moths are less able to distinguish between enantiomers of α-pinene than different odors (e.g., phenylacetaldehyde versus (−)-α-pinene) in learning

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experiments. The relevance of receptor discrimination of enantiomers and learning ability of the moths in host plant choice is discussed.

Key Words—*Heliothis*, EAG, electrophysiology, α-pinene, learning, preference, cotton bollworm, moth.

INTRODUCTION

The peripheral sensory system of an insect initially detects and filters information from the environment. The information is then used to produce an environmentally relevant response. Some questions that have arisen recently in the literature are whether peripheral receptors can distinguish enantiomers of odors, whether the enantiomers are received on the same receptor, and whether this information can be output in terms of effecting behaviorally driven choices (see Stranden et al., 2002).

The discrimination of enantiomers of a single chemical is thought to provide the insect with a greater amount of information about its environment, and thus enable more relevant host choices (Wibe et al., 1998). Insects use the ratio of enantiomers of pheromones as a relevant source of information, as is seen in the communication system of bark beetles, *Ips pini* (see Lanier et al., 1980; Mustaparta et al., 1980), the scarab beetles *Anomala osakana* and *Popillia japonica* (see Leal, 1996), and the moth, *Lymantria dispar* (see Dickens et al., 1997). Enantiomers have also been shown to be important in host choice of the moths, *Dioryctria abietivorella* (see Shu et al., 1997) and the Eurasian cotton bollworm, *Helicoverpa armigera* (see Hartlieb and Rembold, 1996; Bruce and Cork, 2001; Burguiere et al., 2001; Stranden et al., 2002). The sensory basis, and aspects of the behavioral effect of (−)-germacrene-D have been investigated in *H. virescens* (Røstelien et al., 2000; Mozuraitis et al., 2002; Stranden *et al*., 2003). However, the role of experience or learning has not been considered. Learning plays an important role in host plant location by insects (Papaj and Prokopy, 1989). Rather than having fixed behavioral patterns, the attraction of an insect to a plant can be modified according to previous experience. Such learning behavior is thought to allow the insect to respond appropriately in variable environments (Stephens, 1993). In *H. armigera*, learning in adults has been demonstrated in feeding behavior (Hartlieb, 1995; Cunningham et al., 1998a) and in oviposition behavior (Cunningham et al., 1998b). An understanding of the role learning plays in host location is important if we are to improve control techniques that rely on modifying the behavior of the adult moth (Cunningham et al., 1999). Knowledge of the role of learning behavior also extends to our understanding of perception of enantiomers.

Here, we investigate how the enantiomers of another common host plant chemical, α-pinene, are detected by the peripheral sensory receptors of *H. armigera*. We determine if these can be discriminated behaviorally, and whether learning can affect this behavior. We also investigate whether behavioral responses

to enantiomers reflect intensity of antennal response—an assumption inherent in the literature (see Stranden et al., 2002).

METHODS AND MATERIALS

Electrophysiology

Insects. *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) were obtained as pupae from the Queensland Department of Primary Industries, Toowoomba. The culture has been maintained for over 100 generations, with regular injections of wild stock. The moths were kept at constant temperature (23 $\pm$ 1°C) and exposed to a natural light cycle. On emergence, moths were individually placed into 50 ml plastic containers and given unlimited access to water. Female moths were tested between 2 and 5 d of age.

Test Chemicals. The (−) and (+) forms of α-pinene were obtained from Aldrich Chemical Company. Our own determination of optical purity (chiral GC) indicated that both were 95% ee (enantiomeric excess). Absolute purity (GC) for both was greater than 99%.

Electroantennograms (EAGs). This technique was used to assess the summed receptor potentials of the olfactory receptor neurons and to determine response profiles across receptor cell fields. The method used follows Hull and Cribb (2001a) with minor changes: The glass capillaries were filled with a physiological saline (Chen and Friedman, 1975); the antenna was cut off at the base, and secured on Blu-Tack [Bostik (Australia) Pty. Ltd.]. A drop of physiological saline was placed over the base of the antenna to prevent desiccation. The indifferent electrode was inserted into the base of the antenna. The tip of the recording electrode was cut so that it could be placed over the tip of the antenna—which was not cut—as adequate electrical contact could be made this way.

Odor Delivery. Humidified analytical grade compressed air was continuously blown over the moth at a rate of 400 cm^3/min, with the nozzle for the air-stream placed 1 cm from, and directly in front of, the antenna. The tube carrying the air flow was 3 mm internal diam teflon tubing, connected to a glass nozzle (same internal diameter). Test odor samples were taken as saturated vapor, at room temperature, using gas-tight syringes: different volumes of the same concentration were tested (25, 100, 400, 800, 1600, 3200 μl). They were manually injected into the air-stream through a rubber septum, 8 cm from the delivery point. Injection time was between 0.5 and 1.0 sec.

Experimental Procedure. In all experiments the responses were compared to a standard of 400 μl saturated hexanol vapor (equating to 12 nmol at 20°C). The method of standardization, as well as the establishment of the saturating volumes, followed that of Hull and Cribb (2001a). Chemicals were tested as binary mixtures, using 800 μl saturated vapor of each form (800 μl saturated vapor equates to

130 nmol at 20°C of α-pinene for either enantiomer), to determine receptor neuron types. The order of presentation of the mixture series and the chemicals within a mixture series were randomized.

Mixture experiments were analyzed using paired sample *t* tests. Initially a one-way test was conducted to determine if the response to the mixture of chemicals was greater than the response of the larger of the two individual chemicals (i.e., a summating response). If the response to the mixture was summating, then a two-way test was conducted to determine if the response to the mixture was equal to the calculated additive response of the two individual chemicals (i.e., fully summating). Before analysis, the mean result of stimulation with a control injection of clean air was subtracted from the antennal response in both the dose response and mixture experiments. To eliminate the possibility that some or all of the signals were artefacts due to electrode potentials (see Kafka, 1970), control experiments with dead antennae were conducted. The moths were frozen at approximately (−) 20°C. After removal from the freezer, they were allowed to return to normal room temperature before the antennae were tested as above with both (+)- and (−)-α-pinene.

Single Unit Electrophysiology. This technique was used to record the nerve impulses from individual receptor cells and determine their response profiles: responses from two receptors were achieved. Methodology followed Hull and Cribb (2001b) with these modifications: the excised antenna was secured onto Blu-Tack adhesive, and a drop of saline placed over the base to prevent desiccation. The indifferent electrode was inserted into the base of the antenna. The nerve impulses were counted for the first 0.5 sec of stimulation (using Syntech AutoSpike V. 3.1).

Behavior

Insects. Pupae were sexed and female moths were placed into a separate holding cage (200 × 150 × 150 mm) until eclosion. Newly emerged adult females were transferred to sealed 120 mm diam (×6 cm height) plastic containers 2 hr before sunset each day in order to obtain discrete age groups. Moths were deprived of food until used in experiments. Adult moths were kept in a laboratory at 25°C under ambient light conditions. Female moths were tested between 3 and 5 d of age.

Wind Tunnel Trials. Dual-choice preference tests were carried out in a wind tunnel with a central Perspex flight chamber measuring 1600 × 650 × 650 mm (see Cunningham et al., 2004, for details). A laminar flow of clean air was circulated through the flight chamber at 0.7 m/sec (as measured at the center of the chamber using a fan system).

Preference Tests. Procedures for conditioning and testing moths in the wind tunnel have been described in detail previously (Cunningham et al., 2004). Odor sources (lures hereafter) were created by inserting an absorbent cotton wool plug

to a depth of 25 mm below the wide end of a 145 mm glass pipette. An amount of 2 μl of either (+)- or (−)-α-pinene were pipetted onto the cotton wool no more than 15 min before the start of each trial. The narrow end of the pipette was pushed into a block of floral foam (Smithers-Oasis, South Aus.), positioning the odor source at a height of 145 mm above the floor of the wind tunnel. Testing was conducted immediately after conditioning (i.e., approx 1 min). To test the preference of moths for either the (+)- or (−)-α-pinene enantiomer, lures were placed 300 mm apart at the upwind end of the wind tunnel. Smoke tests (using titanium tetrachloride) showed that at a wind speed of 0.7 m/sec these plumes remained separate within the wind tunnel. Two perspex wedges positioned in the downwind end of the wind tunnel brought the odor plumes together at a distance of 800 mm from the lures and left a 200 mm gap through which the odors were directed into the rear portion of the flight chamber.

Moths having previously undergone one of three treatments [associative conditioning with either (+)- or (−)-α-pinene, or no conditioning] were allowed to relocate into the downwind end of the wind tunnel before odor lures were placed into position. Preference for a particular odor was seen as a characteristic upwind flight pattern in the odor plume to within 100 mm of a lure. Once a lure had been approached, the odor type was recorded and the test terminated.

If moths failed to approach either lure within a 5 min period, the preference test was terminated. The position of the feeding lure and odor source in the conditioning trials (centrally placed, 325 mm from either wall) differed from the position of either lure in the preference trials (200 mm from either the right-or left-hand-side wall) so that learning the position of the feeding lure would not influence the choice of lure in the test. The position of each lure (i.e. nearest to the right- or left-hand wall of the chamber) was allocated randomly throughout the experiment to avoid positional biases. The volatile used in conditioning treatments was alternated throughout the experiment.

Associative Conditioning Treatments. Associative conditioning trials were used to determine whether learning of one enantiomer would lead to a preference for that enantiomer in a dual-choice test. The ability to learn to prefer one enantiomer would imply that moths can distinguish between the (+)- and (−)-α-pinene forms. Feeding sites were constructed similarly by plugging the end of a glass pipette with a cotton wool wick that had been soaked in 25% w/v sucrose solution. This second pipette was placed into the same block of floral foam, such that the sucrose wick was situated 2 cm downwind from the lure. New feeding sites and lures were used in each experiment.

Conditioning trials commenced by placing an individual moth on the sucrose wick and allowing a 30 sec feeding bout. Feeding was identified as contact of the extended proboscis with the sucrose wick. In this way, the moth fed approximately 2 cm downwind from the lure. After 30 sec, the moth was removed with a wooden toothpick and placed 400 mm directly downwind from the lure/feeding site. Moths

were then allowed to fly freely back to the feeding source. Upon contact with the sucrose wick, the moth was allowed to feed for a further 20 sec, and then returned to the downwind starting position. This process was repeated until moths had been given a total of four feeding visits in the presence of the volatile; one initial 30 sec feed and 3 × 20 sec return feeds. This procedure has previously been shown to lead to associative conditioning in male *H. armigera* (Cunningham et al., 2004).

Conditioning Trials Using Phenylacetaldehyde. Previous studies using male *H. armigera* moths in an identical experimental design have shown that associative learning of odors leads to a strong preference for the learned odor (Cunningham et al., 2004). To quantify the ability of moths to learn to distinguish between enantiomers of α-pinene, we compared changes in preference for (−)-α-pinene in the above preference test [(−)-α-pinene vs. (+)-α-pinene)] with a learning trial comparing (−)-α-pinene with an alternative floral odor. In the latter test, we compared the odor preferences of female moths conditioned on (−)-α-pinene with moths conditioned on the single floral odor phenylacetaldehyde (90% purity Sigma-Aldrich reagents) in a dual-choice preference test using (−)-α-pinene and phenylacetaldehyde lures (2 μl per lure). Phenylacetaldehyde is a floral volatile to which wild *H. armigera* are naturally exposed and is a well known noctuid attractant that can be learned by male *H. armigera* moths in the laboratory (Meagher, 2001; Cunningham *et al.*, 2004). Conditioning trials and preference testing procedures for this experiment were identical to those described for the α-pinene enantiomer trials.

Innate Preference Treatment. We used unfed female moths with no previous exposure to either α-pinene enantiomer to determine the innate odor preferences. Adult moths were placed into individual sealed (120 mm diam) plastic pots upon emergence and kept until testing at 3 to 4 d old. Preference to (+)- or (−)-α-pinene was determined using the dual-choice testing procedure described above.

Statistical Analysis. Data were analyzed using generalized linear modelling techniques (McCullagh and Nelder, 1989) in the GLIM statistical package (Crawley, 1993). Choice test outcomes were analyzed as proportions, with the number of moths selecting a particular odor as the response variable and the total number of moths selecting either host as the binomial denominator. Binomially distributed error variances were assumed and a logit link function employed. Hypothesis testing was carried out using the χ^2 test on differences in deviance. Treatment order was randomized to prevent any biasing that may have related to night of testing.

RESULTS

Electrophysiology

Dose–Response Curves. For the (+)- and (−) forms of α-pinene, the (−) form gave larger responses at all of the tested concentrations (Figure 1). The

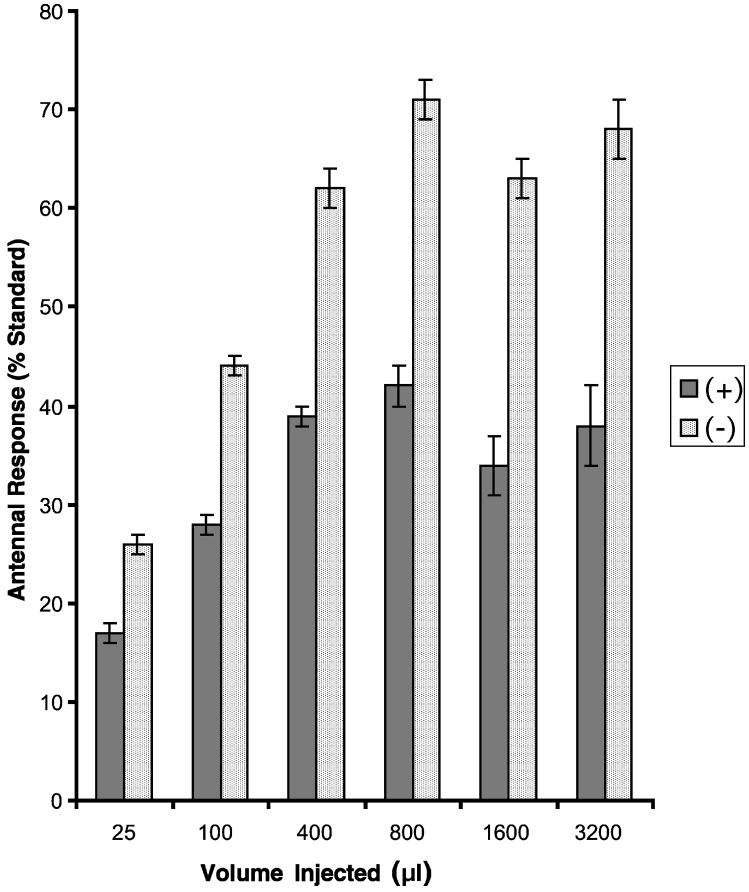

FIG. 1. Dose–response curves of female *H. armigera* antennae in response to (+)- and (−)-α-pinene (each data point represents the mean EAG response from 10 individuals ± one standard error).

saturation point for both forms was reached by 400 μl ($N = 10$). No signals were detected in the control experiments, showing that the signals recorded were solely due to physiological activity within the antenna.

Mixture Experiments. The lack of an additive effect (Figure 2) indicates that the two isomers are being detected by the same sensory receptor cell. The response to the mixture of the two isomers was compared to the larger response of the individual chemicals; the (−) form. The response to the mixture was not larger than the response to the minus form (one-way t test between the mixture and the (−) form, $t = 1.419$, $df = 9$, $P = 0.0948$, $N = 10$).

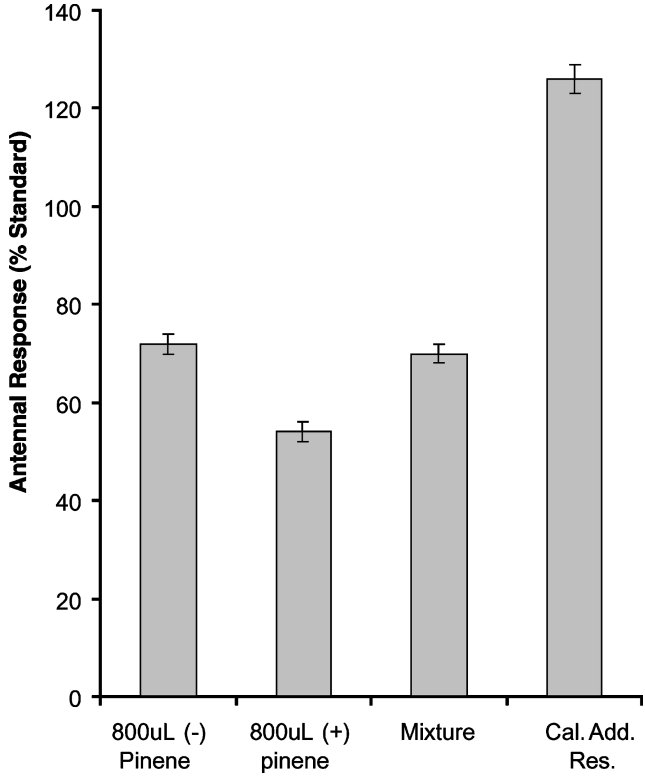

FIG. 2. Results of the mixture experiments between the (+) and (−) enantiomers of α-pinene. The EAG responses represent the mean response ± one standard error ($N = 10$). Cal. Add. Res. = calculated additive response.

Single Unit Electrophysiology. Dose responses for both enantiomers were obtained from two cells. In both cases the cell responded to the (+) and (−) forms: examples are shown in Figure 3. The responses of the cells to the two enantiomers were similar. The cells responded in a dose-dependent fashion and produced up to 37 impulses for the (−) form and up to 38 for the (+) form. One consistent spike height was present, indicating one cell type only was responding. There were no instances of double-height spikes, which would have indicated that two or more cells with a similar spike height were present.

Behavior

We tested the enantiomer preference of 84 female moths from three treatments. Once trained, most moths responded to a lure in preference tests (a total

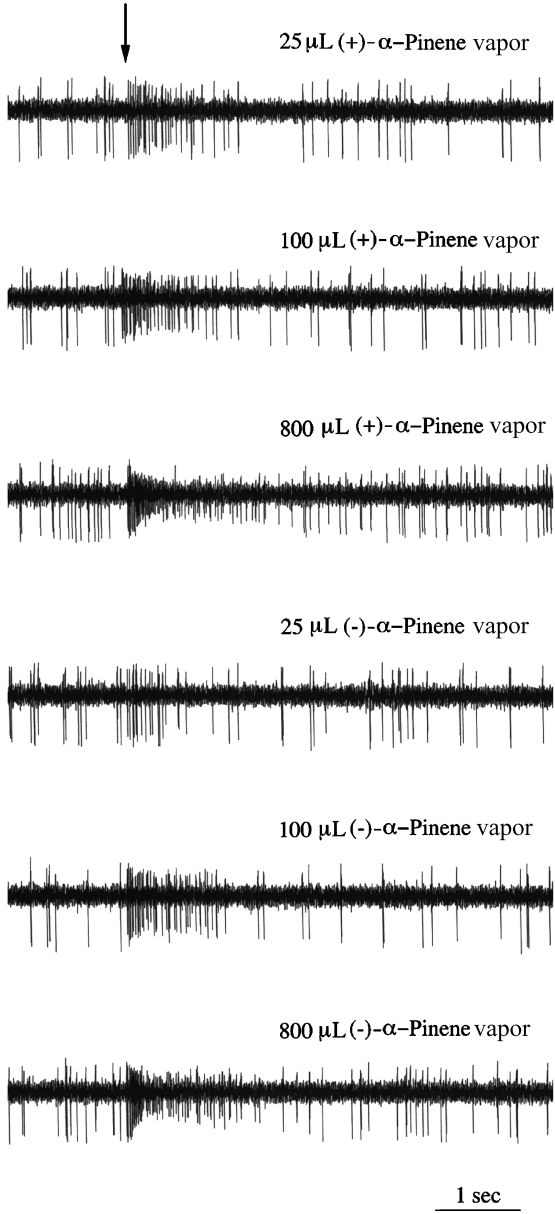

FIG. 3. Electrophysiological responses from a female *H. armigera* sensory cell in response to stimulation with various concentrations of (+) and (−)-α-pinene vapor. Arrow indicates the point of stimulus injection.

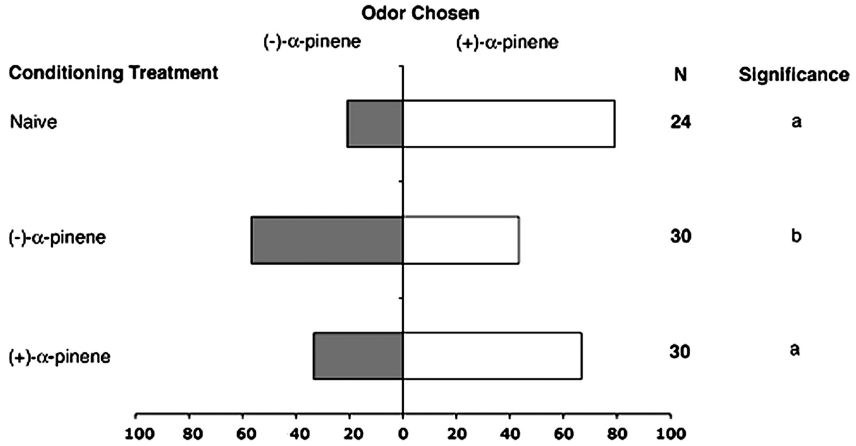

FIG. 4. Percentages of moths selecting either (+)- or (−)-α-pinene in dual-choice preference tests. Treatments with common letters are not significantly different ($P > 0.05$). Different letters denotes a significant difference ($P < 0.01$) as determined by G tests.

of 74 moths were trained in order to achieve 60 completed trials). In dual-choice preference tests (Figure 4), treatment groups showed significant differences in enantiomer preference ($G = 7.832$, $df = 2$, $P < 0.05$). Moths with no odor conditioning showed an innate preference for (+)-α-pinene ($\chi^2 = 8.708$, $df = 1$, $P < 0.005$). This preference displayed by naïve moths was not significantly different from the preferences of moths conditioned on (+)-α-pinene ($G = 1.057$, $df = 1$, $P > 0.05$). However, we found a significant difference in preference between moths conditioned on the (−) enantiomer compared to naïve moths and moths conditioned on (+)-α-pinene ($G = 6.776$, $df = 1$, $P < 0.01$).

Moths were conditioned on either the single floral odor phenylacetaldehyde or (−)-α-pinene in a preference test using these two odors. This test ($N = 24$ moths) showed a significant difference as a result of odor conditioning using these two odors (Figure 5). A strong learning effect was seen: all moths (12/12) trained on phenylacetaldehyde showed a preference for this odor compared with 1/12 moths trained on (−)-α-pinene (i.e., 11/12 moths showed a preference for the (−)-α-pinene lure). This significant difference between treatments ($G = 26.22$, $df = 1$, $P < 0.001$) demonstrates that when given two distinct odors, moths show strong differences in preference.

We compared the changes in preference as a result of experience in (−)-α-pinene vs. (+)-α-pinene trials with tests using phenylacetaldehyde vs. (−)-α-pinene. Moths trained and tested on the latter two odors showed a significantly stronger learning effect compared to moths trained on two enantiomers

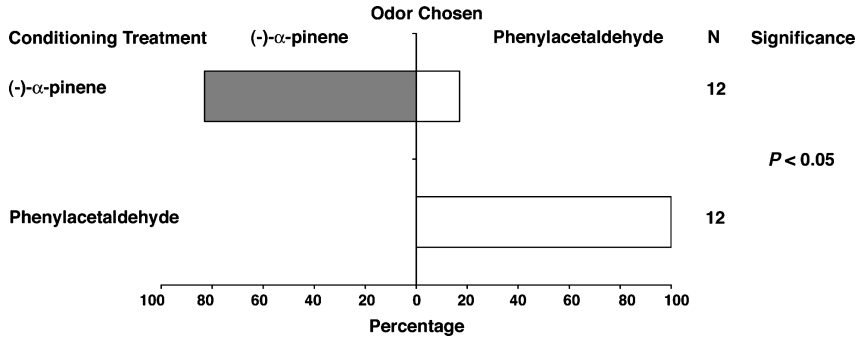

FIG. 5. Percentages of moths selecting (−)-α-pinene and phenylacetaldehyde in a dual-choice preference test. Significant difference ($P < 0.05$) as determined by G test.

of the same odor ($G = 12.31$, $df = 1$, $P < 0.001$). This outcome suggests that although learning changed preferences in both these experiments, the ability to learn the difference between different enantiomers of the same odor was lower than when different odors were learned.

DISCUSSION

α-Pinene is a monoterpene (10-carbon) compound given off by several host plant species of *H. armigera* (Rembold et al., 1989) and has been shown to play a role in behavioral attraction of the female moths toward artificial lures (Rembold et al., 1991). Our electrophysiological results indicate that the two enantiomers of α-pinene are detected by the same receptor cell dendrites: when mixtures of chemicals at saturating levels are used, a response to the mixture that is higher than either of the individual chemicals indicates separate receptor cells are being used (Borst, 1984; Hull and Cribb, 2001a). Because no additive effect was found in EAG experiments, the same cells are likely to receive both enantiomers. Single unit recording confirms this hypothesis. Such a result is consistent with that of Stranden et al. (2002), who found that the enantiomers of a different plant chemical, the sesquiterpene (15-carbon) germacrene-D, were also received by the same receptor cells. The initial dose–response EAG experiments showed that the (−) form of α-pinene produced a higher level of activation of the receptor cell dendrites. This was not mirrored in the single unit recordings where responses appeared similar for (−) and (+) forms, however the small number of receptors directly recorded from does not necessarily provide the average response across the sensillar field: for this information the EAG data are more reliable. The most likely explanation for a higher average response to the (−) form is that the (+) form does not bind as efficiently with the molecular receptor in many of the sensilla;

although less efficiency in other steps of the transduction process such as transport to the receptor site cannot be discounted. A difference in electrophysiological response to the enantiomers of germacrene-D was also found (Stranden et al., 2002). However, this difference was seen only in single unit recordings so the average response for germacrene-D across the sensillar field of *H. armigera* is not yet known.

The behavioral experiments demonstrate that associative learning of (−)-α-pinene leads to an increased preference for this enantiomer compared to the (+) enantiomer and moths without experience of these odors. This provides evidence that *H. armigera* can distinguish between the enantiomers of α-pinene despite their being received on the same receptor. If moths could not differentiate between these two enantiomers, we would not expect any change in enantiomer preference as a result of experience. Moths conditioned on (+)-α-pinene did not show a change in preference relative to moths with no experience. The most likely explanation for this is that the higher innate preference for the (+)-α-pinene enantiomer overshadowed any learning effect. This effect has been seen in studies on odor learning in male *H. armigera* (Cunningham et al., 2004).

The innate preference for one enantiomer is greater than for the other, but the results are counterintuitive. The moths show an innate behavioral preference for the (+) form of α-pinene that provides the smaller physiological signal in EAGs. This result shows that behavioral decisions are not necessarily based simply on the largest physiological response of the receptors, and once again indicates the complexity of the decision-making process. For the studies using germacrene-D, Mozuraitis et al. (2002) only tested the behavioral response to the enantiomer that gave the larger electrophysiological response (in single unit recordings). Our results suggest that further studies with the (+) form of germacrene-D need to be undertaken.

An important outcome from our study is that moths can change their response to enantiomers as a result of experience: in other words, they can learn to discriminate in favor of an enantiomer. This occurred when moths were able to increase their response to the (-) form over the innate response. One possible explanation for the way in which the same receptor might discriminate different enantiomers is *via* a concentration effect. This deserves further investigation. Although discrimination was achieved, the ability to learn to distinguish between the (+) and (−) enantiomers was low when compared to learning to distinguish (−)-α-pinene from the single odor phenylacetaldehyde, suggesting a behavioral interaction in response between enantiomers. Stranden et al. (2002) hypothesize that separate receptor neuron types for enantiomers will be needed if an insect is to be able to distinguish a plant based on differences in enantiomeric composition of specific compounds. Our data indicate that this is unlikely to be the case for α-pinene. However, response spectra for this single receptor type might vary from neuron to neuron.

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WINTER CHERRY BUGS FEED ON PLANT TROPANE ALKALOIDS AND DE-EPOXIDIZE SCOPOLAMINE TO ATROPINE

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Abstract—The winter cherry bug colonizes the *Duboisia leichhardtii* tree, which is a rich source of scopolamine. It consumes the tropane alkaloids atropine and scopolamine. Quantitative analysis revealed that the ratio of scopolamine to atropine in the winter cherry bug (0.46) was far from that found in the leaves of the host plant (7.20). To elucidate whether the winter cherry bugs selectively excrete or decompose scopolamine, they were fed scopolamine and/or atropine together with sucrose. They took up scopolamine as well as atropine, and converted scopolamine into atropine.

Key Words—*Acanthocoris sordidus*, Coreidae, *Duboisia leichhardtii*, Solanaceae, plant-insect interaction, tropane alkaloid, atropine, scopolamine, de-epoxidation.

INTRODUCTION

Medicinal plants produce various secondary metabolites, most of which have protective roles against physical threats like UV radiation, and defensive functions against herbivores and pathogenic microbes (Gershenzon, 2002). A typical example would include the tropane alkaloids atropine and scopolamine, the production of which is restricted to certain genera of Solanaceae such as *Atropa*, *Datura*, *Duboisia*, *Hyoscyamus*, and *Scopolia*. Generally, atropine and scopolamine exert effects on the neurotransmitter acetylcholine, and act as neurotoxins in insects as well as mammals (Evans, 1996).

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The interaction between pyrrolizidine alkaloid-producing plants and insects has been extensively studied, and the mechanism by which insects adapt to pyrrolizidine alkaloids, as well as the insects that use these alkaloids, have been well elucidated (Hartmann, 1999). However, scant data on the tropane alkaloids have been made available, except for that on *Datura stramonium*. In the case of *D. stramonium*, Shonle and Bergelson (2000) showed that insects exhibit individual selection for two alkaloids, atropine and scopolamine, using specialist flea beetles as well as generalist herbivores such as the cucumber beetle. However, they did not report how these insects reacted to tropane alkaloids.

Here, we report for the first time that the winter cherry bug (*Acanthocoris sordidus*, Coreidae) adapts to tropane alkaloid-producing plants, taking in and accumulating these alkaloids in their bodies. In addition, feeding experiments have clarified that *A. sordidus* de-epoxidizes scopolamine to atropine.

METHODS AND MATERIALS

Insect and Plant Material Collected from the Field. In addition to the perennial plants *Physalis alkekengi* L. var. *francheti* Hort. (Solanaceae) and *Bupleurum scorzoneraefolium* Willd. var. *stenophyllum* Nakai (Apiaceae), the tree *Duboisia leichhardtii* F. Muell (Solanaceae), which is indigenous to Australia and New Caledonia, was cultivated as a specimen at the Medicinal Plant Garden, attached to the Biomedical Sciences, Nagasaki University, Nagasaki, Japan. Adult insects colonizing *D. leichhardtii*, as well as *P. alkekengi* and *B. scorzoneraefolium*, were identified as the winter cherry bug (*Acanthocoris sordidus* Thunberg) (Coreidae) and *Graphosoma rubrolineartum* Westwood (Pentatomidae), respectively. The winter cherry bugs were collected from their host plants and used for the feeding experiments and alkaloid analysis. *G. rubrolineartum* insects were also collected from *B. scorzoneraefolium* plants for preliminary feeding experiments. *D. leichhardtii* leaves harvested from the tree were used for alkaloid analysis. The materials were dried at 50°C before analysis.

Alkaloid Feeding. *Acanthocoris sordidus* and *Graphosoma rubrolineartum*, caught in the field, were reared overnight for a preliminary trial. Both insects (10 each) in different jars (9.5 cm diam, 18.5 cm high) were supplied an artificial diet on triple layers of filter paper (5.5 cm diam) in petri dishes (6.0 cm diam) containing 3 ml of aqueous solution, which was mixed with 400 mg of sucrose and 200 mg of atropine sulfate. In subsequent feeding experiments, only *Acanthocoris sordidus* were reared for 4 days. They were divided into 3 groups (10 per group) and were fed by the method described above, but with different dietary solutions. The 3 ml of aqueous solution mixed with 0.5 mmol sucrose (171 mg), and 0.25 mmol alkaloids, were as follows: 1) atropine sulfate alone (0.25 mmol, 87 mg); 2) both atropine sulfate (0.125 mmol, 44 mg) and scopolamine hydrobromide (0.125 mmol, 55 mg); 3) scopolamine hydrobromide alone (0.25 mmol, 110 mg). Atropine

sulfate and scopolamine hydrobromide were purchased from Merck. During the 4 days of rearing, 1 ml of distilled water was added every day to the filter papers to avoid dessication.

Alkaloid Extraction and Analysis. Alkaloids were extracted from *A. sordidus* and *D. leichhardtii* and identified by using HPLC as described by Sauerwein and Shimomura (1991), except for the use of a Finepak Sil C18S (JASCO, 4.6 × 150 mm) as the column and homatropine hydrobromide (Extrasynthesis, France) as the internal standard. Alkaloids on the filter papers were recovered in MeOH (10 ml) by shaking overnight and then extracted as described above. Atropine and scopolamine were confirmed by GC-MS, as reported previously (Kitamura et al., 1991).

RESULTS AND DISCUSSION

In the summer of 1996, insects belonging to Heteroptera had increased dramatically and were causing serious damage to agricultural products in Japan. At about the same time, we found an insect forming colonies on the *Duboisia leichhardtii* tree. Since then, this phenomenon has continued from time to time in summer, with the insects on the surface of *Duboisia* stems, but not leaves, their proboscis with stylets stuck in the bark, where they remain for a couple of days. The insects seem to chew the phloem sap from the bark, as is commonly found in insects belonging to Hemiptera possessing a proboscis. As *Duboisia* phloem sap contains tropane alkaloids together with sucrose (Kitamura et al., 1993), we recognized that this insect of necessity must be able to adapt to tropane alkaloids. The insect was identified as the winter cherry bug (*Acanthocoris sordidus* Thunberg) (Coreidae).

To confirm the uptake of alkaloids, we analyzed atropine and scopolamine in the insects as well as in *Duboisia* leaves by using TLC, HPLC, and GC/MS, finding that plant alkaloids were present in the winter cherry bugs. Interestingly, the ratio of scopolamine to atropine in the winter cherry bugs was very low (0.46), compared to that in the host plant (7.20) (Table 1). We speculated that this decrease in the scopolamine ratio might be due to selective decomposition and/or excretion of scopolamine by the insect.

To test this idea, a laboratory feeding experiment was employed. As the preliminary step, *Acanthocoris sordidus* on the tree *Duboisia leichhardtii* and *Graphosoma rubrolineartum* on the perennial plant *Bupleurum scorzoneraefolium* were caught in the field and reared in jars, independently. They were fed on wet filter papers containing a diet solution mixed with atropine and sucrose. After overnight rearing, 100% of the *A. sordidus* were alive and fine, whereas 40% of the *G. rubrolineartum* had died. During rearing, it was observed that many *A. sordidus* visited the filter paper frequently and touched their proboscis to the filter paper, sometimes staying there for over 30 min. In contrast, no visits by

TABLE 1. ALKALOID CONTENTS IN WINTER CHERRY BUGS (*Acanthocoris sordidus*) FED VARIOUS DIETS AND IN THEIR HOST PLANT, *D. leichhardtii*

No.	Sample	Alkaloid content (μmol/g dry wt.)		
		scopolamine (S)	atropine (A)	ratio (S/A)
1	*D. leichhardtii* leaves	9.43	1.31	7.20
2	*A. sordidus* on *D. leichhardtii**	0.21	0.46	0.46
3	*A. sordidus* fed with A	<0.01	45.26 ± 7.73	n.z.
4	*A. sordidus* fed with A/S	<0.01	30.66 ± 10.92	n.z.
5	*A. sordidus* fed with S	<0.01	36.83 ± 13.87	n.z.

No. 1–2, sample from field; No. 3–5, feeding experiments ($n = 3$, means $\pm$ standard deviation). n.z., nearly zero.
*Alkaloid content in an individual insect was too scant to analyze.

G. rubrolineartum were recorded. The reason why 40% of *G. rubrolineartum* died is not clear, but it is possible that they touched the toxic solution or that the rearing conditions were somehow not suitable. In any event, it was confirmed that *A. sordidus* has adapted to atropine and can survive on the artificial diet.

In further feeding experiments, only winter cherry bugs colonizing the non-tropane alkaloid-producing plant *Physalis alkekengi* were used, so as to avoid confusion between alkaloids from the field plants and from the artificial diet. The artificial diet, containing scopolamine and/or atropine, together with sugar, was supplied as an aqueous solution on the filter paper. During rearing, as in the preliminary feeding experiment, many *A. sordidus* visited the filter papers frequently, and extended their proboscises to the filter paper containing the alkaloid diets. There were no detectable differences in feeding behavior between the insects with different diets. After 4 days, it was determined which alkaloid had been retained in their bodies as well as which remained on the filter papers. Surprisingly, only atropine was detected in the winter cherry bugs, regardless of the diet (Table 1). An individual bug retained atropine at a level between $0.96 \sim 0.43$ μmol (0.55 ± 0.22 μmol) after 4 days of rearing. From the filter papers, the same alkaloids that were supplied in the various diets, were recovered. We did not detect, however, either scopolamine in the atropine diet or atropine in the scopolamine diet.

The results indicate that the winter cherry bug took up and accumulated tropane alkaloids, and de-epoxidized scopolamine to atropine (Figure 1). This answers the question of why the amount of scopolamine was much lower than atropine in the winter cherry bugs on *D. leichhardtti*, which produce more scopolamine than atropine. The bugs reared with the scopolamine diet converted all the scopolamine to atropine. In addition, the amount of alkaloid accumulated in the winter cherry bugs reared with the artificial diet was nearly a hundred times higher than that collected from the field. This insect concentration may depend on alkaloid availability in the diet. The laboratory feeding experiment, at least,

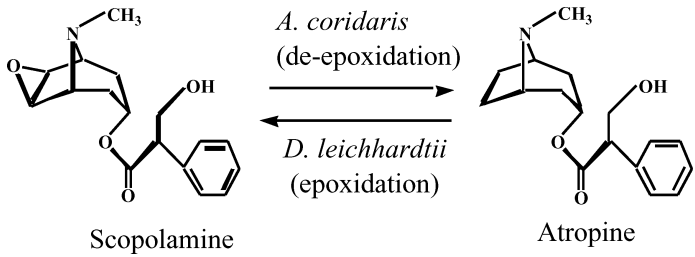

FIG. 1. Conversion between scopolamine and atropine.

indicates that the winter cherry bug possesses a high sequestration potential for atropine.

In the case of the Colorado potato beetle, glycoalkaloids seem to be excreted, rather than sequestered or metabolized (Armer, 2004). In contrast, the pyrrolizidine alkaloid senecionine is chemically detoxified to N-oxide by insects that have adapted (Hartmann, 1999). Similarly, the winter cheery bugs de-epoxidize scopolamine and convert it to atropine. Since both scopolamine and atropine are efficient anticholinergic agents, a detoxification mechanism appears unnecessary. Scopolamine is more toxic than atropine, however, because it is more hydrophobic and hence able to pass the brood-brain barrier that controls entry into the central nervous system.

Duboisia plants biosynthesize scopolamine from atropine by epoxidation, but the winter cherry bugs convert scopolamine into atropine by de-epoxidation. While tropane alkaloid-producing plants are defended against herbivores, the winter cherry bugs have apparently circumvented this barrier, and may even utilize these neurotoxins to protect themselves from predators. This supports the idea of co-evolution (Rausher, 2001), as suggested by Shonle and Bergelson (2000). Further study is necessary to determine whether the winter cherry bugs lose the alkaloid during subsequent feeding on an alkaloid free diet. In addition, the ecological and biological functions of the de-epoxidized scopolamine by the winter cherry bugs, and the subsequent effects on their predators, are of sufficient interest to pursue the details.

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EVIDENCE THAT PETROMYZONTID LAMPREYS EMPLOY A COMMON MIGRATORY PHEROMONE THAT IS PARTIALLY COMPRISED OF BILE ACIDS[1]

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Abstract—This study examined whether the larval pheromone employed by adult sea lamprey (*Petromyzon marinus*) to locate spawning streams and known to be at least partially comprised of bile acids is also employed by other lamprey species. Both production and release of lamprey-specific bile acids, and sensitivity to them were examined in a wide variety of species. High pressure liquid chromatography and electrospray ionization/mass spectrometry (ESI-MS) found gallbladders from 10 species of European and North American lamprey to contain large quantities of petromyzonol sulfate (PS) together with much smaller quantities of allocholic acid (ACA) and petromyzonol (P). Evaluation of holding waters from three of these species using ESI-MS found all to contain large quantities of PS and lesser quantities of ACA in similar ratios. Electro-olfactogram recording from the olfactory systems of three parasitic lamprey species found all to detect PS and ACA with high sensitivity. Behavioral studies using migratory adult sea lamprey found them to be attracted to the odors of heterospecific larvae as well as conspecific larvae, both of which contained similar amounts of PS and ACA. Finally, adult silver lampreys (*Ichthyomyzon unicuspis*) were also found to be attracted to the odor of larval sea lamprey. Together, these results demonstrate that PS and ACA are commonly produced and released by larval petromyzontid lampreys and likely used as part of a common evolutionarily conserved pheromone. This scenario is reasonable because lampreys share similar larval and spawning habitat requirements, and their larvae derive no apparent benefit from producing compounds that serve as an attractant for adults.

Key Words—Pheromone, migration, lamprey, Petromyzontidae, petromyzonol sulfate, allocholic acid, petromyzonol, evolution, species-specificity.

[1]For ease of discussion and to be consistent with existing literature on biliary compounds, we use the term "bile acid" to describe all biliary steroids even though some such as petromyzonol are alcohols.

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INTRODUCTION

The sea lamprey, *Petromyzon marinus*, is an ancient cartilagenous fish that spends its larval life in freshwater streams, metamorphoses and emigrates into oceans or large lakes where it parasitizes other fish, grows and matures, and then migrates back into streams to spawn (Applegate, 1950; Purvis, 1980). Sea lampreys are dispersed great distances as parasites and make no attempt to return to their natal streams (Bergstedt and Seelye, 1995). Instead, a variety of evidence has shown that adult sea lamprey locate streams using innately recognized odorous cues, a critical component of which is a pheromone released by stream-resident larval lampreys (Sorensen and Vrieze, 2003). Not only are adults unable to locate streams efficiently if their olfactory sense is impaired (Sorensen and Vrieze, 2003), but the capture rate of adults entering streams correlates with the presence of larvae living in them (Moore and Schleen, 1980). Further, larval sea lamprey odor is attractive to conspecific adults at low, biologically relevant concentrations (Vrieze and Sorensen, 2001). It seems reasonable that adult sea lampreys have evolved to employ this strategy because the presence of larvae correlates with the presence of nursery habitat and by default, spawning habitat as well.

The sea lamprey migratory pheromone is known to be at least partially comprised of bile acids produced by larval conspecifics. Several studies have shown that larval sea lamprey produce and release significant quantities of the sulfated 24-carbon bile acid, petromyzonol sulfate (PS: 3α, 7α, 12α, 24-tetrahydroxy-5α-cholan-24-sulfate), and its probable precursors, allocholic acid (ACA: 3α, 7α, 12α -trihydroxy-5α-cholan-24-oic acid) and petromyzonol (P: 3α, 7α, 12α, 24-tetrahydroxy-5α-cholan) (Li et al., 1995; Polkinghorne et al., 2001). Enzyme-based high performance liquid chromatography (HPLC) suggests that larvae release PS at about three times the rate of ACA, which is released in greater amounts than P (Polkinghorne et al., 2001). Further, electrophysiological recording has shown that the adult olfactory system detects both PS and ACA with extreme specificity and sensitivity (picomolar thresholds; Li et al., 1995; Li and Sorensen, 1997). Petromyzonol does not appear important to the pheromone because its detection threshold is relatively high (about 10^{-8} Molar (M); Li and Sorensen, 1997). Lastly, and most importantly, migratory adult sea lampreys are attracted to low (10^{-10} M) concentrations of mixtures of PS and ACA in both laboratory and raceway mazes although not as strongly as to larval holding water (Bjerselius et al., 2000; Vrieze and Sorensen, 2001). Recent HPLC fractionation studies of extracts from larval holding water suggest that there is at least one unknown component in the pheromone that is bile acid-like (Sorensen et al., 2003).

Lamprey pheromone systems are of interest for several reasons. First, the sea lamprey is a significant pest; it invaded the Great Lakes and is now the subject of a substantial control program that is exploring pheromones for use in control (Li et al., 2003; Sorensen and Vrieze, 2003; Twohey et al., 2003). Second, several

species of coastal lamprey are threatened, and pheromones are being considered to enhance their spawning runs (Close et al., 2002; Yun et al., 2003). Third, lampreys occupy a position near the base of the vertebrate evolutionary tree. There are 34 species and 8 genera of lampreys in the Northern Hemisphere, and all are members of the family Petromyzontidae (according to Gill et al., 2003). Interestingly, a species thought to be ancestral to this lineage, the silver lamprey, *Ichthyomyzon unicuspis*, is available for testing. Although all members of the Petromyzontidae start life as stream-dwelling larvae which filter-feed, some (such as the sea lamprey) metamorphose into a parasitic phase that disperses downstream (often to oceans or lakes), while others lack a parasitic stage and mature directly from the larval stage. Adults of both parasitic and nonparasitic species move upstream to spawn when mature, and in the case of the former these movements can take the form of long-distance migrations (Hardisty and Potter, 1971a; Malmqvist, 1980). With the exception of the sea lamprey and *Caspiomyzon wagneri* from the Caspian Sea, all genera of petromyzontid lamprey contain at least one parasitic species and one nonparasitic species, with the latter being derived from the former (Potter, 1980; Docker et al., 1999; Gill et al., 2003). Although it was recently shown that larval Pacific lamprey [*Entosphenus tridentata* (formerly in the genus *Lampetra*)], and western brook lamprey (*Lampetra richardsoni*) produce and likely release PS, it is not clear whether these species also produce and release ACA and P (Yun et al., 2003). Further, it is unknown whether any other lamprey species produce and release specific mixtures of PS, ACA, and P, or whether any species other than the sea lamprey detects these cues for the purpose of migration. This question is of some interest because where studied in fish, bile acid production has been found not to vary below the taxonomic level of family (Hoshita, 1985), calling into question both how specific a pheromone partially comprised of bile acid(s) might be, and how it could have evolved.

The present study sought to determine whether the bile acid(s) used as a migratory pheromone by the sea lamprey represent cues employed just by this species or common metabolic byproducts produced by related species. Here, we asked four questions: 1) Are PS, ACA, and P produced by larvae of a wide range of European and North American petromyzontid lamprey species? 2) Do these species also release PS, ACA, and P? 3) Do the olfactory systems of other lamprey species detect lamprey bile acids with similar sensitivities and specificities as the sea lamprey? 4) Are migratory adult lampreys attracted to the odor of larval lampreys of other species known to contain similar suites of bile acids?

METHODS AND MATERIALS

Are PS, ACA, and P Produced by a Wide Range of Petromyzontid Lampreys? Larval lampreys from five of the eight genera of petromyzontid lamprey including

TABLE 1. LAMPREYS EXAMINED IN THIS STUDY

Scientific name	Common name	Range
Petromyzon marinus	Sea lamprey	Atlantic coasts of North America & Europe
Entosphenus tridentata	Pacific lamprey	Pacific coasts of North America & Asia
Lethenteron appendix	American brook lamprey	Great Lakes Basin/Eastern North America
Lampetra richardsoni	Western brook lamprey	Pacific coast of North America
Lampetra fluviatilis	European river lamprey	Atlantic coast of Europe
Lampetra planeri	European brook lamprey	Atlantic coast & interior Europe
Ichthyomyzon fossor	Northern brook lamprey	Great Lakes Basin
Ichthyomyzon unicuspis	Silver lamprey	Great Lakes Basin
Ichthyomyzon castaneus	Chestnut lamprey	Great Lakes Basin
Ichthyomyzon gagei	Southern brook lamprey	Interior North America

parasitic and nonparasitic species were collected from sites across the Great Lakes Basin, the Pacific and Atlantic coasts of North America, and the Atlantic coast of Europe by using electroshocking and trapping (Table 1). This group included representatives of the most derived (*Lampetra*) and basal (*Ichthyomyzon*) genera. Between two and seven individuals of 10 species were euthanized (overdose of MS222; Argent Chemical Laboratories, WA) and their gallbladders (site of bile acid storage) removed. Bile acids were extracted following the protocol of Locket and Gallaher (1989), which employs methanol-activated reversed-phase C18 bonded-phase cartridges (Sep-pak Plus, Waters Corp., Milford, MA), dried under a stream of nitrogen, and reconstituted in 2 ml of methanol. Bile acids found in the gallbladder extracts were characterized using an established enzyme-based HPLC technique that specifically detects 3α-hydroxy steroids including most bile acids (see Polkinghorne et al., 2001). Results were confirmed with mass spectrometry. Briefly, for analysis by HPLC, 5% of each extract was injected onto a reversed-phase C18 column (Nova-pak C18, 4 μm column; Waters Chromatography Division, Milford, MA) and eluted with a gradient of acetonitrile and ammonium dihydrogen phosphate (25 mM, pH 7.8). This eluate was passed though a column containing 3α-hydroxysteroid dehydrogenase so that any H^+ produced by oxidation of 3α-hydroxy bile acids could be monitored by a fluorescence detector (excitation: 340 nm; emission: 420–650 nm). For the purposes of quantitation, 10-point calibration curves running from 0.0 to 3.0 μg were created for PS, ACA, and P (Toronto Research Inc., Toronto, Canada). The detection threshold was defined as the lowest concentration that could be included to maintain linearity ($r^2 > 0.95$). Two non-lamprey bile acids (1-μg hyocholic acid, 1-μg lithocholic acid) were added to all samples to confirm retention times.

To confirm the identities of bile acids, a subset of 12 samples from 4 species was reanalyzed by electrospray ionization-mass spectrometry (ESI-MS). In each

case, 5 μl aliquots of extract were injected into an ion trap mass spectrometer (LCQ Classic, Finnigan, MAT) equipped with an electrospray ionization source [ion trap operated in the negative ion mode with a spray voltage of 5 kV; sheath gas was 99% pure nitrogen at 60 psi; sheath fluid was 50:50 20 mM triethylamine:acetonitrile (v/v)]. To quantify ACA we used selected reaction monitoring because its molecular ion (407.4 m/z [M-H]$^-$) consistently fragmented at a normalized collision energy of 30% to produce an ion at 380.9 m/z that could be quantified using an 8-point calibration curve (0.1–15.0 ng). To quantify PS and P, we employed selected ion monitoring because the molecular ions of these compounds did not fragment. We examined profiles of 10 m/z units centered around their molecular ions (PS: 473.4 m/z; P: 393.4 m/z), measuring peak areas at the appropriate locations (molecular ion ± 1 m/z) and comparing these values to 8-point standard curves (0.1–15 ng). Curves were generated in methanol and gallbladder extracts to confirm accuracy.

Are PS, ACA, and P also Released by Petromyzontid Lamprey Species? We examined bile acid release from the three species found in greatest abundance in the Great Lakes Basin: sea lamprey, American brook lamprey (*Lethenteron appendix*), and northern brook lamprey (*I. fossor*). The first species is parasitic, the last two, nonparasitic. Larvae were collected by electroshocking rivers whose species compositions were well known (J. Slade, Ludington Biological Station, Ludington, MI), and brought to the Hammond Bay Biological Station (Millersburg, MI) in aerated containers. There they were weighed, sorted by species based on their morphometrics (Vladykov and Kott, 1980), and placed into three separate 350-l tanks to create densities of about 400 g larvae per tank (811 sea lamprey, 402 American brook lamprey, 418 northern brook lamprey). A fourth tank was maintained with aerated lake water and sand only as a control.

Tanks were maintained and sampled following established protocols (see Polkinghorne et al., 2001). Briefly, tanks were provided with a 10-cm layer of sand (in which larvae burrow), and supplied with aerated Lake Huron water (5 l/min at 10°C). Every 3 d, larvae were fed by turning incoming water off and adding bakers yeast (which larvae consume; Fleischmann's, St. Louis, MO). Water flow was resumed 24 hr later for 12 hr to flush uneaten yeast, after which it was shut off again for 12 hr, and then samples of water collected. The control tank lacking larvae was treated in the same manner although less yeast was added. After collection, water samples were filtered through paper filters (Whatman International Ltd., Maidstone, England), and extracted using methanol-activated reversed-phase C18 bonded-phase cartridges (1 l per cartridge). Loaded columns were washed with 12-ml 20% methanol, 6-ml water, 6-ml hexane, and eluted with 5-ml methanol. This protocol has an extraction/recovery efficiency of approximately 90% for PS, ACA, and P (Polkinghorne et al., 2001). Electrospray

ionization-mass spectrometry (ESI-MS) was used to quantify bile acid content in extracted holding water after PS, ACA, and P had been isolated by HPLC fractionation. This was accomplished using the protocols employed for gallbladder analysis and collecting three 5-min fractions centered around the retention times of PS, ACA, and P. Collected fractions were then reextracted through C18 Sep-Pak columns, dried under nitrogen, and reconstituted in 100-μl methanol (recovery rate was 64% $\pm$ 4%), and aliquots quantified using ESI-MS.

Do the Olfactory Systems of Petromyzontid Species Detect Lamprey Bile Acids with Similar Sensitivities and Specificities as the Sea Lamprey? To test whether other lampreys might detect the same bile acids as sea lamprey, we employed electro-olfactogram (EOG) recording, a multiunit extracellular technique that measures summed receptor generator potentials (Sorensen and Caprio, 1998). Procedures were identical to those developed for the sea lamprey (Li et al., 1995). We tested migratory silver lamprey and Pacific lamprey, species that we could obtain in abundance and whose adults were large enough to permit EOG recording. Both species were captured in traps during their migratory period; silver lamprey came from tributaries of the Mississippi River (IA), and Pacific lamprey from the Umatilla River (OR). Briefly, individual lampreys were anesthetized in 1:1,000 MS222, immobilized with an injection of gallamine triethiodide (Sigma, 150-mg/kg body weight), and placed onto a stand where their gills were irrigated with 12°C well water. Their olfactory epithelia was then exposed, irrigated with well water, and responses recorded differentially using Ag/AgCl electrodes (type EH-1S; WPI, Sarasota, FL) bridged to glass capillaries and amplified by a DC amplifier (Grass P16, Warwick, RI). One electrode was placed on the surface of the head and the other positioned just above the olfactory epithelium to yield maximal responses. If we were unable to record responses of at least 0.75 mV to our standard [10^{-5} Molar (M) L-arginine; Sigma, St. Louis, MO], the fish was not used. We tested 5-sec pulses of 10^{-8} M PS, ACA, P, and seven other bile acids dissolved in trace amounts of methanol (Toronto Research, Inc., Toronto, Canada; Sigma, St. Louis, MO; Table 2). Analysis followed established protocol (Li and Sorensen, 1997) in which response magnitudes were measured from the baseline to the peak of each displacement. The magnitude of any responses elicited by the methanol control was subtracted, duplicate responses averaged, and this value expressed as a percentage of the most recent response elicited by the standard. These data were then plotted along with data previously collected for the sea lamprey (Li et al., 1995; Sorensen, unpublished data) so that the species could be compared.

Do Adult Lamprey Species Respond to Heterospecific Larval Odors also Known to Contain PS and ACA? Behavioral responses of migratory adult sea lamprey (1999) and silver lamprey (2002) to larval odor were tested following established protocols (Vrieze and Sorensen, 2001). We tested larval holding waters known to contain bile acids (samples of these waters were analyzed in Experiment 2) believing tests of whole odor to be the most direct test of pheromone specificity.

TABLE 2. BILE ACID ODORANTS FOR ELECTRO-OLFACTOGRAM RECORDING

Abbreviation	Common name	Chemical name
PS	Petromyzonol sulfate	3α,7α,12α,24-Tetrahydroxy-5α-cholan-24-sulfate
ACA	Allocholic acid	3α,7α,12α-Trihydroxy-5α-cholan-24-oic-acid
TLS	Taurolithocholic acid 3-sulfate	3α-Hydroxy-5β-cholan-24-oic-acid-*N*-(2-sulfoethyl)-amide-3-sulfate
TCA	Taurocholic acid	3α,7α,12α-Trihydroxy-5β-cholan-24-oic-acid-*N*-(2-sulfoethyl)-amide
P	Petromyzonol	3α,7α,12α, 24-Tetrahydroxy-5α-cholan
HDC	Hyodeoxycholic acid	3α,6α-Dihydroxy-5β-cholan-24-oic-acid
LCA	Lithocholic acid	3α-Hydroxy-5β-cholan-24-oic-acid
HCA	α-Hyocholic acid	3α,6α,7α-Trihydroxy-5β-cholan-24-oic-acid
TUD	Tauroursochenodeoxycholic acid	3α,7β-Dihydroxy-5β-cholan-24-oic-acid-*N*-(2-sulfoethyl)-amide
CA	Cholic acid	3α,7α,12α-Trihydroxy-5β-cholan-24-oic-acid

Migratory adult sea and silver lampreys were obtained from traps located in tributaries of the Great Lakes and placed into Lake Huron water at the Hammond Bay Biological Station (MI) for at least 3 d prior to testing. For testing, we employed 1.8 m $\times$ 9.0 m two-choice mazes (Vrieze and Sorensen, 2001) supplied with Lake Huron water (425 l/min) into which 4 l/min of water from a stream that lacked lamprey was introduced [Nagel Creek, MI (river water synergizes responses to larval odor; Vrieze and Sorensen, 2001)]. Each maze had an upstream release section and a downstream choice section with a central channel and two side channels (Figure 1). Tests were conducted after sunset because lampreys are nocturnal. Trials started with odor introduction, 5 min after which a group of four lampreys was released from one of four holding cages located in either the upstream section (sea lamprey) or the center channel (the smaller silver lamprey). These lampreys were given 20 min to respond, removed, and then another group tested after a 15 min break. Responses of adult sea lamprey were tested to dilute holding waters from larval sea lamprey, American brook lamprey, and northern brook lamprey. In each case, water from larval tanks was introduced down one arm, while the same amount of water from a control tank lacking larvae was added to the other. For the "high" concentration, holding waters were diluted 10,000 times; this was equivalent to placing a single larval lamprey into 500-l water for 1 hr. For the "low" concentration, we diluted holding waters 100,000 times; this concentration of larval odor simulated that present in many lamprey streams (Polkinghorne et al, 2001). Stimulus side was changed nightly. Mazes were illuminated by overhead infrared floodlights and equipped with low light cameras and video recorders. For analysis, videotapes were reviewed, and the positions of each group of four lampreys were noted every 30 sec for 20 min. Percent time in the side-channel

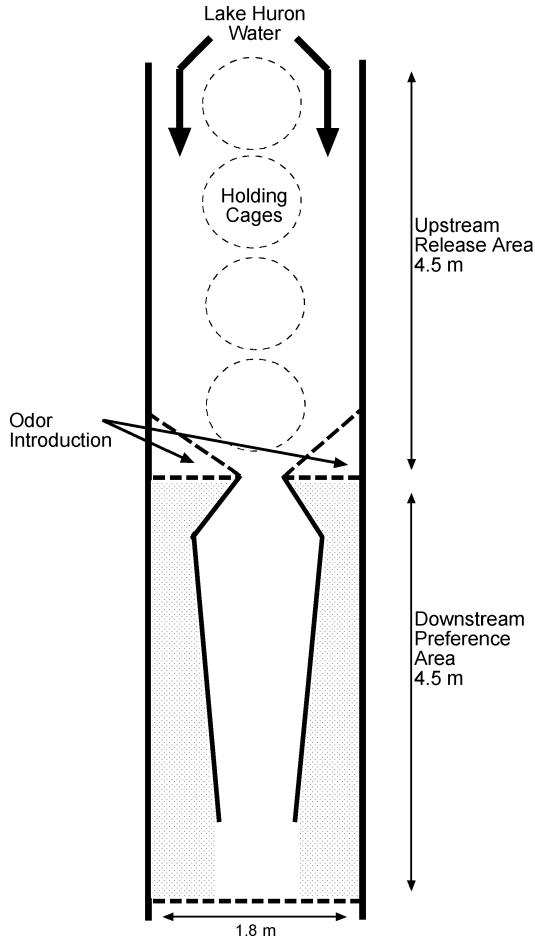

FIG. 1. Representation of the maze used to test behavioral preferences of adult lampreys. Dashed lines denote plastic mesh stretched across the maze. Test odors were introduced immediately upstream of each side channel. Shading shows the area of the side channels in which lamprey distribution was quantified. Adult sea lampreys were released from cages in an upstream region and moved downstream where they encountered odors and then moved into the side channels.

with larval odor was then calculated for each group and arc-sine transformed to meet the assumptions of parametric analysis, and compared to 50% (e.g., no preference) using a two-tailed one-sample t test (see, Vrieze and Sorensen, 2001). Means and 95% confidence intervals were then back transformed for presentation.

RESULTS

Bile Acid Production. Analysis of larval gallbladders found them to contain between one and three clearly discernable 3α-hydroxy-steroids, all of which closely matched retention times of known lamprey bile acid standards (Figure 2). The largest peak for all 10 species had a retention time that matched PS. Quantities of putative PS ranged from 289 μg/gallbladder for western brook lamprey to 65.1 μg/gallbladder in northern brook lamprey, with gallbladder size varying by a factor of two (Table 3). In contrast, ACA was only consistently measurable in sea lamprey and American brook lamprey, and then in low quantities (2–5 μg/gallbladder). Finally, P was also only consistently measured in two species, and then at very low levels (<5 μg/gallbladder). Using HPLC, the detection thresholds for all three bile acids was about 0.03 μg/injection (0.60 μg/gallbladder).

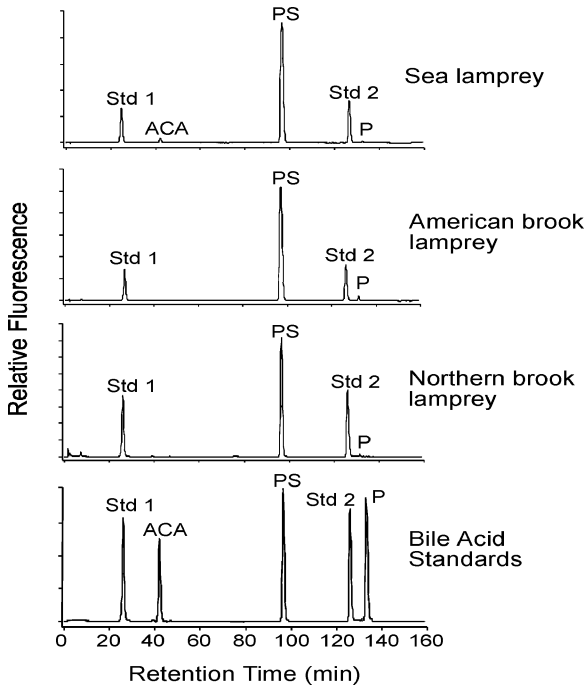

FIG. 2. Representative HPLC chromatograms of gallbladder extracts from larval sea lamprey (*P. marinus*), American brook lamprey (*Lethenteron appendix*), and northern brook lamprey (*I. fossor*) and standards ("std 1" = 1 μg hyocholic acid; "std 2" = 1 μg lithocholic acid; ACA = 1 μg allocholic acid; PS = 1 μg petromyzonol sulfate; P = 1 μg petromyzonol).

TABLE 3. BILE ACID CONTENT IN LARVAL LAMPREY GALLBLADDERS AS MEASURED BY HPLC

Species	*N*	Gallbladder mass (mg)	Total bile acid present in gallbladder (mean ± SE) PS (μg)	ACA (μg)	P (μg)
Sea lamprey	7	4.7 ± 1.3	102.6 ± 17.6	5.0 ± 1.9	0.7 ± 0.4[a]
Pacific lamprey	4	5.5 ± 1.3	267.3 ± 83.1	N.D.	N.D.
American brook lamprey	7	7.9 ± 3.0	218.1 ± 36.5	1.8 ± 0.9[a]	4.1 ± 0.9
Western brook lamprey	4	8.0 ± 3.3	289.0 ± 28.7	N.D.	N.D.
European river lamprey	5	N.A.	106.6 ± 21.5	N.D.	0.1 ± 0.1[a]
European brook lamprey	4	N.A.	225.8 ± 56.0	N.D.	1.0 ± 1.0[a]
Northern brook lamprey	7	5.7 ± 2.4	65.1 ± 12.5	N.D.	0.3 ± 0.2[a]
Silver lamprey	4	2.8 ± 0.3	79.1 ± 11.7	N.D.	N.D.
Chestnut lamprey	5	4.8 ± 0.8	73.9 ± 14.2	N.D.	1.6 ± 0.5
Southern brook lamprey	2	4.2 ± 1.3	167.8 ± 54.7	N.D.	N.D.

Note. N.D. = Not detectable (5% gallbladder content injected; detection limit was 30 ng/injection). N.A. = Not available. *N* = number of individual gallbladders tested.
[a]Less than half of individual animals had detectable quantities.

Mass spectrometry confirmed the presence of all three bile acids in all gallbladders and permitted quantitation. Detection limits were about 20 ng/ml/injection (2 ng/gallbladder) for PS and ACA, and 80 ng/ml/injection (8 ng/gallbladder) for P. Petromyzonol sulfate was evident in large quantities in the gallbladders of all four species (Figure 3), and in quantities similar to those measured by HPLC (Table 4). Much smaller quantities of P (less than 1 μg/gallbladder) were also measurable in all samples from all four species. Allocholic acid consistently fragmented into four peaks at 361.3 m/z, 380.9 m/z, 394.4 m/z, and 407.4 m/z, of which 380.9 was the largest and easiest to quantify (Figure 4). Using this peak, we estimated

TABLE 4. BILE ACID CONTENT IN LARVAL LAMPREY GALLBLADDERS AS MEASURED BY MASS SPECTROMETRY

Species	*N*	Gallbladder mass (mg)	Total bile acid present in gallbladder (mean ± SE) PS (μg)	ACA (μg)	P (μg)
Sea lamprey	3	3.1 ± 1.5	85.1 ± 16.9	5.7 ± 2.6	0.5 ± 0.2
European river lamprey	3	N.A.	88.3 ± 5.7	0.1 ± 0.04	0.11 ± 0.1
Northern brook lamprey	3	4.8 ± 2.8	54.4 ± 9.7	0.2 ± 0.04	0.2 ± 0.1
Southern brook lamprey	3	4.1 ± 0.9	144.1 ± 27.4	0.07 ± 0.05	0.07 ± 0.04

Note. Five percent gallbladder content injected; detection limit per sample was 50 pg/injection; N.A. = not available; *N* = number of individual gallbladders tested.

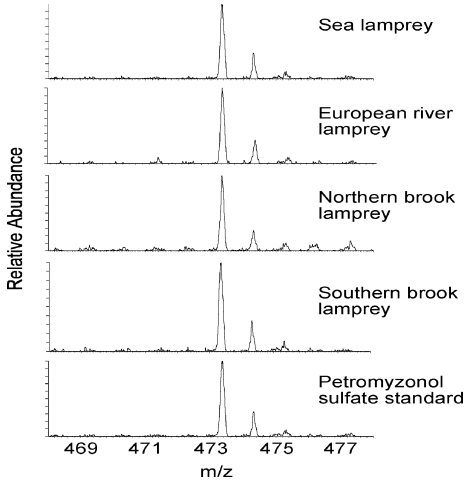

FIG. 3. Representative mass spectrometry chromatograms of gallbladder extracts from larval sea lamprey (*P. marinus*), European river lamprey (*Lampetra fluviatilis*), northern brook lamprey (*I. fossor*), southern brook lamprey (*I. gagei*), and petromyzonol sulfate standard (PS; 0.4 ng). Chromatograms show profiles of 10 m/z units centered around 473.4 m/z, the $[M\text{-}H]^-$ ion for PS.

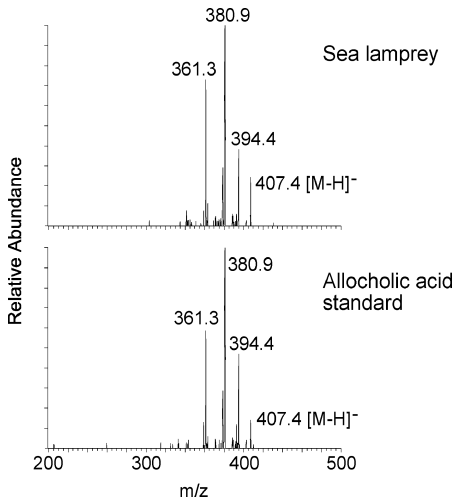

FIG. 4. Mass spectra of a gallbladder extract from a larval sea lamprey (*P. marinus*) and allocholic acid standard (ACA; 0.6 ng) from 200 to 500 m/z. The precursor ion is 407.4 m/z $[M\text{-}H]^-$.

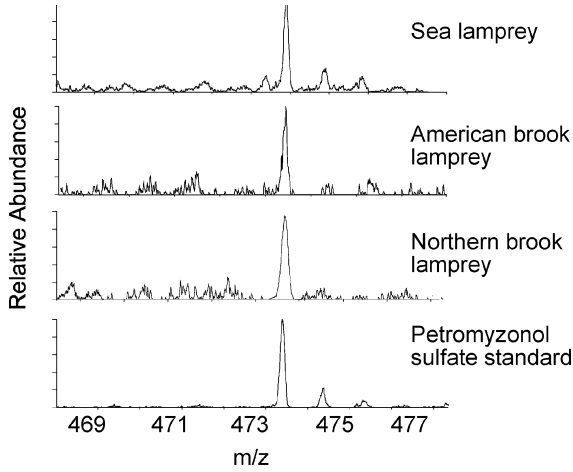

FIG. 5. Representative mass spectrometry chromatograms of extracted holding water from sea lamprey (*P. marinus*), American brook (*Lethenteron appendix*), northern brook lamprey (*I. fossor*), and petromyzonol sulfate standard (PS; 0.4 ng). Chromatograms are profiles of 10 m/z units centered around 473.4 m/z, the $[M\text{-}H]^-$ ion for PS.

sea lamprey gallbladders contain nearly 6 μg/gallbladder, while all gallbladders of the other species contained between 0.1 and 1.0 μg. The ratio of PS to ACA to P was fairly consistent across species ranging from 1.0 : 0.48 : 0.006 for the sea lamprey to 1.0 : 0.0002 : 0.0009 for European river lamprey (Table 4).

Bile Acid Release. Petromyzonol sulfate was identified in the release waters of all three larval species by MS; it consistently had a profile centered at 473.4 m/z with little background (Figure 5). All three species released about 2 ng/lamprey/hr (Figure 6). Allocholic acid was also found in holding water from all species

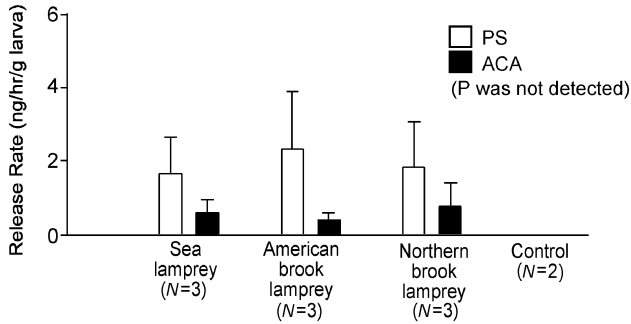

FIG. 6. Mean rates (±standard error) at which petromyzonol sulfate (PS) and allocholic acid (ACA) were released for larval sea lamprey (*P. marinus*), American brook (*Lethenteron appendix*), and northern brook lamprey (*I. fossor*). *P* was not detected in any samples.

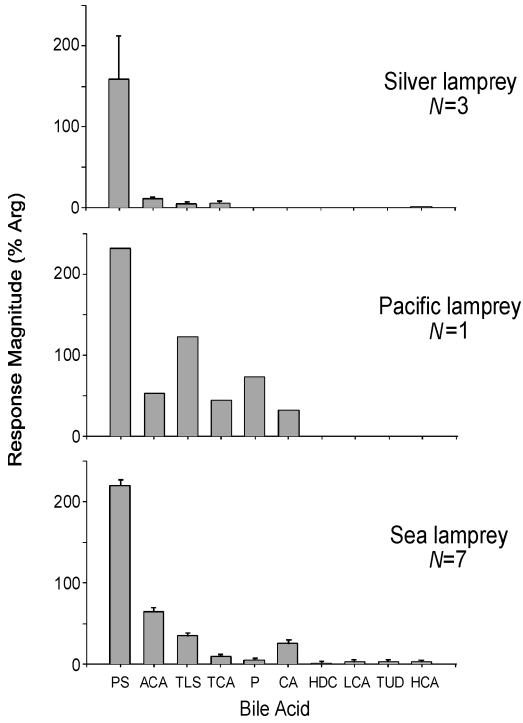

FIG. 7. Average relative electro-olfactogram (EOG) responses (± standard error) recorded from migratory adult silver lamprey (*I. unicuspis*; $N = 3$), Pacific lamprey (*E. tridentata*; $N = 1$), and sea lamprey (*P. marinus*; $N = 7$; data from Li et al., 1995; P. W. Sorensen, unpublished). EOG response magnitudes to 10 bile acids at a concentration of 10^{-8} M are expressed relative to that of the standard (10^{-5} M L-Arginine).

by using selective reaction monitoring (data not shown). Petromyzonol was not detectable in the holding water of any species. The ratio of PS : ACA in release waters was about 3:1 for all species (Figure 6).

Olfactory Sensitivity. While usable EOG responses were easily recorded from three of the five silver lampreys we tested, only one of the seven Pacific lampreys yielded responses to the standard that met our minimum requirement. Silver and Pacific lamprey detected PS with great sensitivity; they elicited responses that were about twice the size of the standard, a value similar to that for the sea lamprey (Figure 7). Both species also detected ACA; they elicited responses about 10% the size of the standard in silver lamprey and 25% of the standard in the single Pacific lamprey that we obtained responses from, again similar to sea lamprey. Silver lamprey did not respond to P, and only responded to two of the other seven bile acids [taurolithocholic acid 3-sulfate (TLS), taurocholic acid (TCA)], and then with little

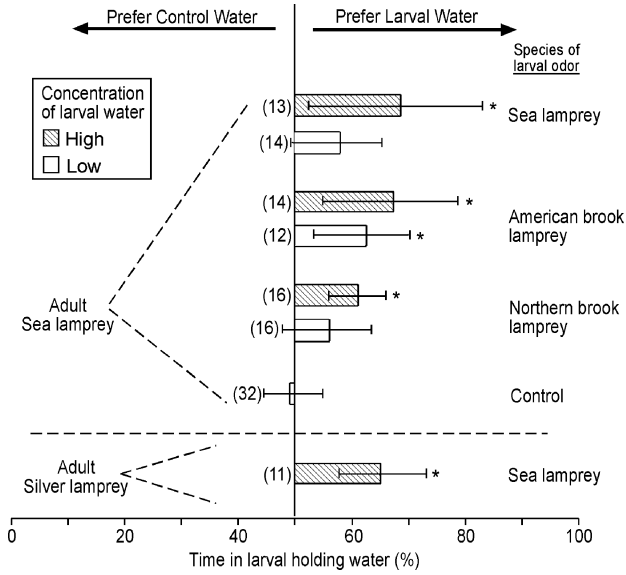

FIG. 8. Mean percent time (±95% confidence intervals) spent by adult sea and silver lampreys in larval sea lamprey (*P. marinus*), American brook (*Lethenteron appendix*), and northern brook lamprey (*I. fossor*) holding waters in the test maze. Two concentrations were tested—"high": 1 g larvae/500 l/hr; and "low": 1 g larvae/5000 l/hr. The control test employed water from the holding tank lacking larvae down each channel and analyzed preferences for the right side vs. the left. Numbers in parentheses denote the number of trials for each experiment. Means were compared to a no-preference value of 50% using a student's *t* test; $^{*}P < 0.05$.

sensitivity. Finally, EOG responses of the Pacific lamprey to P, TLS, TCA, and cholic acid (CA) were relatively modest and similar to those of the sea lamprey.

Behavioral Responsiveness. Adult sea lamprey were attracted to the odor of all three species of larval lamprey at the high concentration, spending 60–70% of their time in these odors ($P < 0.05$; Figure 8). Adult sea lamprey also spent more time in the low concentration of holding water from all three larvae than control water, although only responses to American brook lamprey holding water were significant ($P < 0.05$; Figure 8). Finally, silver lampreys were also attracted to larval sea lamprey holding water ($P < 0.05$).

DISCUSSION

The results of this study demonstrate that the production and release of PS and ACA by larval sea lamprey is not a specialized trait but rather a common

one to many, and perhaps all, members of the family Petromyzontidae. Further, the ability to detect these bile acids is shared by adults of at least three parasitic petromyzontid species, two of which are also strongly attracted to the odor of larvae of other species. Although the assortment of species and scenarios we tested was not ideal, it did include parastic and nonparasitic species from five of eight extant genera, including the most basal (*Ichthyomyzon)* as well as the most derived (*Lampetra*; Potter and Hilliard, 1987; Docker et al., 1999; Gill et al., 2003). Our results provide no clear evidence for species-specific differences in the production of bile acids by lampreys. Thus, although larval sea lampreys appear to accumulate more ACA in their gallbladders than the other species, MS clearly demonstrated that all species also produce ACA and P. Yun et al.'s (2003) inability to measure P and ACA in Pacific lamprey gallbladders can likely be explained by their use of less sensitive techniques. Similarly, there was no compelling evidence for species-specific specializations in bile acid release mechanism; all three species released PS:ACA at a ratio of about 3:1, a value similar to that previously described by Polkinghorne et al. (2001) for the sea lamprey. In conclusion, the bile acid pheromone appears common to all North American lampreys.

The absolute rate of PS and ACA release estimated by this study supports the possibility that these compounds function as pheromones with release rates falling within the range needed to produce picomolar (detectable) concentrations of bile acids in Great Lakes' streams. Notably, the rates we estimated were only slightly less than those we have measured before (Polkinghorne et al., 2001) but 10–20 times less than those suggested by Yun et al. (2003) for Pacific lampreys using immunoassays. However, the latter estimates may have been confounded by the use of a relatively nonspecific antiserum. Of course, larval holding conditions, temperature, feeding, and health may also influence larval bile acid release rates. Ours is the first study we know of to use a definitive biochemical technique such as MS to measure bile acid release by a fish.

Interestingly, bile acid release did not correlate tightly with the relative quantities of bile acids found in larval gallbladders. Perhaps, as noted in both mammals (Elliott, 1985; Hylemon, 1985) and skates (Fricker et al., 1997), there is selective absorption of particular bile acids (i.e., PS vs. ACA) by the lamprey gut prior to release. Polkinghorne et al. (2001) calculated that less than 1% of all PS produced by larval sea lamprey is released. It is unlikely that intestinal absorption reflects specialization for pheromonal function because PS is the most potent odorant and attractant (Li et al., 1995; Sorensen and Fine, unpublished results). In contrast, absorption of PS by lamprey likely reflects a physiological process that has evolved to limit large-scale loss of this dominant steroid (Elliott, 1985). Pheromonal function, thus, would represent a secondary neural specialization on the part of adults.

Both our EOG recordings and behavioral studies suggested that all adult lampreys respond in similar manners to bile acids and natural larval odors that contain these compounds. EOG response profiles of adult sea and silver lampreys were similar and, while some differences appeared possible for the Pacific lamprey, this may have been an artifact of our having been able to record usable responses from only a single animal. This difficulty was likely caused by deterioration of their physiological condition associated with advanced maturational state (Sorensen et al., 1995). Most importantly, our behavioral studies showed that low, biologically significant concentrations of odor from three species of larval lamprey were strongly attractive to migratory adult sea lamprey and that silver lamprey were attracted to the odor of larval sea lamprey. Our finding that adult sea lampreys are attracted to the odor of other lamprey species native to the Great Lakes is consistent with numerous observations by lamprey control biologists that adult sea lamprey often enter streams which contain high densities of heterospecifics but few conspecifics (personal communication, D. Cuddy, Lamprey Control Centre, Sault Ste. Marie, Canada).

One difficulty with interpreting our results is that we do not yet understand the complete composition of the migratory pheromone. Another is that we were unable to perform extensive behavioral testing using multiple species and their odors to definitively determine cue specificity. Nevertheless, the present study demonstrates that critical bile acid component(s) of this cue are commonly produced, released, and detected by a variety of lamprey species including members of basal and derived genera. Further, we know that adults of at least two species do not distinguish between the odorous cues released by different species, suggesting that all components found in this cue (including the bile acids) are commonly produced and released. That lampreys might employ a set of related bile acid products would be parsimonious because the development of multiple release and detection mechanisms is not required. Indeed, it appears to mirror the situation for fish sex pheromones, which are mixtures of related hormonal products (Sorensen and Stacey, 1999; Stacey and Sorensen, 2002).

That many/all petromyzontid lampreys might employ a similar, relatively unspecialized pheromone containing bile acids to guide migration of parasitic adults seems plausible. Evaluating the ecological and physiological costs of bile acid release to larvae, we can not conceive of any reason why larvae of any species might have evolved to produce and/or release any particular bile acids that might attract adults of any particular species because they derive no apparent benefit from doing so. Similarly, it seems likely that migratory adults of all lamprey species benefit by detecting and employing a generic set of related pheromonal (bile acid) cues because a large number (perhaps all) of them have similar nursery and spawning habitat requirements (Hardisty and Potter, 1971b; Morman, 1979; Maitland, 1980; Manion and Hanson, 1980). Additionally, migratory adults appear to risk little by responding to other species cues, as all petromyzontid

lampreys are nest builders with similar habitat requirements and well developed courtship behaviors that appear to include sex pheromones (Manion and Hanson, 1980; Yamazaki and Goto, 2000; Li et al., 2002); likely they can discern each other's identities and avoid hybridization. Facilitating this scenario is the fact that speciation in lampreys has proceeded in such a manner that generally only a few species of lamprey are sympatric in one locale, many of which are parasitic and nonparasitic species pairs that differ greatly in size (Potter, 1980). Indeed, the natural range of sea lampreys overlaps little with other species. Accordingly, it seems reasonable that the bile acids released by all larval lampreys should serve as part of a long-distance pheromone that universally denotes the presence of suitable spawning and nursery habitat for migratory adult lampreys of many species.

In summary, it appears likely that petromyzontid lampreys evolved to employ PS, ACA, and other possibly related compounds released by their larvae, and that this trait has been conserved through evolutionary time. Although an important driving force behind the use of this cue appears to lie with the migratory adults, it is interesting to speculate that the blind larvae of both parasitic and nonparasitic species, which face similar challenges locating good habitat with adequate food, may also employ this cue to locate each other in streams, and that this function may even pre-date the use of water-borne bile acids to mediate migration. Notably, where studied, larval distributions are discontinuous, suggesting the presence of aggregational cues (Morman, 1979; Morman et al., 1980; Schuldt and Goold, 1980; Fodale et al., 2003). Although final proof regarding the lack of specificity of the sea lamprey migratory pheromone awaits, the answer may not have great bearing on sea lamprey control in the Great Lakes because silver lamprey are the only other Great Lakes species with a lacustrine parasitic phase, and it is much smaller (Vladykov and Kott, 1980). A relatively nonspecific pheromone might be useful because indigenous species of larval lamprey could be nurtured above traps and barriers to lure adult sea lampreys (Sorensen and Vrieze, 2003). A generic lamprey bile acid pheromone also has good potential to enhance runs of endangered and threatened species of indigenous coastal Pacific and European petromyzontid lampreys in their historic spawning habitats (Tuunainen et al, 1980; Close et al., 2002).

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USE OF CHEMICAL COMMUNICATION BY THE SUBTERRANEAN RODENT *Ctenomys talarum* (TUCO-TUCO) DURING THE BREEDING SEASON

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Abstract—Solitary subterranean rodents with a low frequency of direct contact between conspecifics are expected to use chemical communication to coordinate social and reproductive behavior. We examined whether reproductive tuco-tucos *(Ctenomys talarum)* were able to discriminate the reproductive condition, sex, and source population of conspecifics by means of chemical cues contained in urine, feces, soiled shavings, or anogenital secretions. During preference tests in which animals had direct contact with these chemical cues, tuco-tucos were able to determine the reproductive condition of opposite sex conspecifics independent of the source of odor. When only olfactory cues were available, both sexes discriminated reproductive condition of opposite sex individuals using urine. Females were also able to discriminate the reproductive condition of males using soiled shavings. Females spent more time investigating male odors than female odors; except in the case of feces, breeding males spent similar amounts of time investigating male and female odors. No preferences were detected for opposite sex urine from members of an animal's own versus another population. The role of chemical cues in territory defense and breeding performance by this highly territorial subterranean rodent is discussed.

Key Words—Subterranean rodent, odor preference, olfaction, vomeronasal organ, reproductive differences, sexual differences.

INTRODUCTION

Natural selection is expected to favor signals that are transmitted efficiently in a species' typical environment (Hauser, 1998). As a result, a wide array of biotic

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and physical factors may influence the evolution of animal signals (Endler, 1993). Included in this array is the perceiver's ability to detect the signal as well as its current attentional status (Dusenbery, 1992).

Among subterranean rodents, the specialized physical conditions of underground burrows may significantly affect the communication channels used by their occupants (Francescoli, 2000). Because vision is practically useless in dark tunnels and touch is restricted to close-contact situations, the use of chemical and vibrational signals is expected to be enhanced in this habitat, particularly because the latter signals can be transmitted beyond the limits of a burrow system and are thus effective for long distance communication (Francescoli, 2000). Although chemical cues are confined to communication within the limits of a burrow system, they are also expected to be important to subterranean rodents because they may compensate for visual signals (Doty, 1986), and because scents deposited on the substrate (soil) can remain active for relatively long periods in the absence of the sender (Bradbury and Vehrencamp, 1998). At present, however, little is known about the use of odor cues or the mechanisms of chemosensory perception in subterranean rodents. Those studies that have been conducted have focused on the eusocial African mole-rats *(Heterocephalus glaber*: Faulkes and Abbott, 1991, 1993; O'Riain and Jarvis, 1997; *Cryptomys sp*.: Heth et al., 2002) or the solitary blind mole-rat *Spalax ehrenbergi* (e.g., Heth and Todrank, 1995; Heth et al., 1996a, b; Todrank and Heth, 1996; Shanas and Terkel, 1997), allowing limited opportunity to evaluate the importance of chemical cues in life underground.

In general, chemical cues are thought to play an important role in mediating many aspects of rodent reproductive behavior and physiology. Several species can discriminate among odors deposited by opposite sex individuals in different reproductive conditions (Johnston, 1979; Huck et al., 1989) and attractiveness of odor sources may vary across female reproductive states (Lai et al., 1996). Seasonal changes in scent properties and responses to them are dependent on the interaction between gonadal hormones, gender, and photoperiodic history (Ferkin and Johnston, 1993; Ferkin et al., 1994, 1997a). As a consequence, the attractiveness of conspecific odors may vary seasonally, which may reflect temporal changes in social organization and patterns of association. For example, both male and female meadow voles, *Microtus pennsylvanicus*, produce scents that are attractive to the opposite sex during the breeding season. In contrast, during the nonbreeding season, female odors are attractive only to females whereas males produce scents that are not attractive to either sex (Ferkin and Seamon, 1987; Ferkin et al., 1995).

Species with a low frequency of direct interaction between conspecifics are expected to rely on chemical communication to coordinate their social and reproductive behavior. In particular, when aggressive behavior must be overcome to allow mating, odor cues may play an important role in reducing aggression among potential partners and in helping individuals to reach sexual readiness. Given these functions, we expect chemical communication to be important in the solitary

subterranean rodent *Ctenomys talarum* (Rodentia: Octodontidae) commonly known as the tuco-tuco. This species is found along the coast of Buenos Aires province and, possibly Santa Fe province, Argentina (Redford and Eisenberg, 1992). They are highly territorial rodents that live alone (Busch et al., 1989) in underground tunnel systems, which consist of numerous foraging tunnels, a single central tunnel and a nest chamber (Antinuchi and Busch, 1992). Aggression is common between interacting individuals and mating pairs show an elaborate pattern of courtship behavior (Zenuto et al., 2002).

Collectively, these attributes suggest that communication among conspecifics is important in *C. talarum*. A complex vocal repertoire has been identified (Zenuto et al., 2001; Schleich and Busch, 2002) but seismic communication is apparently lacking in this species. Several lines of evidence indicate that chemical signals may be important. For example, scent marking as well as sniffing of urine, feces, and the anogenital area was recorded during territorial and courtship encounters (Zenuto et al., 2001, 2002). The ability to distinguish familiar from novel scents was confirmed via habituation–discrimination tests (Zenuto and Fanjul, 2002). Response to chemical cues appeared to vary between the sexes; during the nonbreeding season, females were attracted to male odors, whereas males spent the same amount of time investigating odors of either sex (Fanjul et al., 2003). *C. talarum* is polygynous (Zenuto et al., 1999), and it is possible that these differences in response to chemical cues are related to intersexual differences in patterns of reproductive competition in this species.

Information encoded in odors derived from the body's normal metabolism may play an important role in mediating reproductive behavior for the highly aggressive and solitary subterranean rodent *C. talarum*. In this study, we tested the general hypothesis that *C. talarum* individuals are capable of discriminating reproductive condition, sex, and population of origin by means of chemosensory cues. Specifically, we predicted that (1) individuals will be able to detect the reproductive condition of opposite sex individuals using olfactory cues (urine, feces, soiled bedding, anogenital secretions), (2) recognition of the reproductive condition of opposite sex individuals will be more generalized (i.e., uses more sources of odor) when individuals have direct contact with the odor source, (3) olfactory cues in urine, feces, and soiled shavings will differ between the breeding and nonbreeding seasons, and (4) individuals will be able to detect interpopulation differences in urine odors produced by reproductive animals of the opposite sex.

METHODS AND MATERIALS

Animals. The breeding season of *C. talarum* extends over 9 months (June–January), during which the highest percentage of pregnancies was recorded in

August, followed by a second post-partum breeding in November (Malizia and Busch, 1991). Adult *C. talarum* (donor individuals) were collected at Mar de Cobo (37° 45′S, 57° 56′W) during both the nonbreeding ("nonreproductive individuals") and breeding season (donor and test "reproductive individuals"). Additional donor individuals were collected at Necochea (38° 33′S, 58° 45′W), during the breeding season. A total of 63 (28 males, 35 females) nonreproductive and 91 (43 males, 48 females) reproductive *C. talarum* from Mar de Cobo served as scent donors and 105 animals (47 males, 58 females) from this population served as test individuals. Twenty-four tuco-tucos (12 males, 12 females) from Necochea served as scent donors. Test animals participated in two (and in a few cases three) experiments; in such cases, there was at least a 1-wk interval between trials. Animals were never exposed to the same odor donors in two different experiments. The participation of test and donor animals was randomly determined for each experiment. Pregnant females were excluded from this study to avoid interfering with embryo development.

All animals were housed individually in plastic cages (42 × 34 × 26 cm) with wood shavings as bedding. They were fed *ad libitum* with carrots, sweet potatoes, lettuce, and mixed grasses. Because *C. talarum* do not drink free water, it was not provided. Nonreproductive donor animals and reproductive donor and test animals were maintained under the light cycle that corresponded to nonbreeding and breeding season, respectively. Temperature was automatically controlled (23 ± 1°C). Individuals were allowed to adapt to captivity for at least 10 d prior to participation in experiments or odor collection. Once odor collection and/or experiments concluded, the animals were released at their place of capture.

Odor Collection. The odor sources used in this study were feces, urine, soiled shavings, and secretions from the anogenital area. Feces and urine were collected from donor animals using a metabolic cage. Urine samples were collected for a period of 24 hr in glass vials containing mineral oil; urine samples were discarded if they were contaminated with fecal material. Similarly, feces were discarded if contaminated with urine. Shavings soiled with urine, feces, and presumably other body secretions and elements of their degradation were collected from the soiled corners of cages, in which the bedding had not been changed for 7 d. Anogenital odor was collected by rubbing a water-moistened cotton swab against that area of an individual's body for 5 sec. Samples were individually collected using plastic bags (soiled shavings) or 1.5 ml eppendorf tubes (urine, feces, anogenital secretions) and stored at −20°C. The scent samples were kept frozen for not more than 6 months and were thawed just before use in an experiment. Disposable latex gloves were used during all procedures to minimize human scent transfer while handling scent samples.

Odor Testing Procedure. Preference tests were used to determine whether male or female *C. talarum* displayed a preference for different odor stimuli. Odor samples were presented to the test animals in glass Petri dishes (50 mm diameter)

covered with wire mesh in order to prevent the animals from coming into direct contact with the sample except during experiment 2, in which scent samples were offered without a mesh barrier so that the test animals could lick or touch the sample. For each odor type, the test animals were exposed simultaneously to two odor samples: the odor of a reproductive individual of the opposite sex and the odor of a nonreproductive individual of the opposite sex (experiment 1). In experiment 2, the same odor types were presented without a wire mesh. In experiment 3, test animals were exposed to odors from same and opposite sex reproductive individuals. Experiment 4 used odors from reproductive individuals of the opposite sex from the same and from a different population as the test animal.

For each test, 50 μl of urine, four fecal pellets, a cotton swab with anogenital secretions, or a full Petri dish of soiled shavings was used. Unscented Petri dishes were also provided in each test as a control. Thus, test animals had three potential stimuli to investigate: odor 1, odor 2, and the odorless control. The odorless control Petri dishes were clearly not attractive to animals, because they spent insignificant time smelling them (overall mean $\pm$ SE: 0.29 $\pm$ 0.09 s). Because the responses to controls were restricted to incidental and very short investigations, the controls were not included in the data analyses.

The experimental apparatus consisted of two acrylic cages (45 $\times$ 30 $\times$ 30 cm) that were connected to each other by an acrylic tube (10 cm wide $\times$ 20 cm long). During preference tests, the test animal was confined for at least 1 hr in one of the cages, which contained soiled shavings from the test animal's own cage. At the start of each trial, the test individual was allowed to enter the second cage, which contained clean shavings and the Petri dishes with the scent samples. Placement of stimulus odors on the left or the right side of the box was randomized to control for possible side preferences by the test animals. For all trials, the amount of time (in sec) that the subjects spent investigating each Petri dish was recorded for 7 min using stopwatches. We recorded that a tuco-tuco was investigating an odor when it appeared to be sniffing the odor sample and/or its nose was within 1 cm of the surface of the Petri dish. After each test, both cages and the tube were carefully washed with tap water and odorless glassware cleaner, wiped with alcohol, and then allowed to air dry to ensure that no odors from the previous trial remained.

Data Analysis. Paired *t*-tests or Wilcoxon matched-pairs signed-ranks tests were used to test for significant differences between the total time spent investigating each stimulus pair. Kruskal–Wallis tests were performed to test the equality of time spent by each sex exploring different odor sources. Post hoc multiple pairwise comparison tests were used to identify specific differences when Kruskal–Wallis tests revealed significant differences in overall patterns of response. Mann–Whitney tests were performed to test the equality of time spent by males and females exploring each odor source. Significant differences were accepted at $P < 0.05$ (two-tailed test). Data are expressed as mean $\pm$ SE.

RESULTS

Experiment 1. The reproductive state of female scent donors affected male preferences for urine and soiled shavings (Figure la, Table 1). Marginally significant differences were detected for feces but no differences were detected for secretions of the anogenital area (Figure la, Table 1). Females spent more time investigating urine from reproductive males, but showed no difference in response when confronted with other odor sources (feces, soiled shavings, and anogenital area; Figure 1b, Table 1). Test females spent more time investigating stimulus odors (both reproductive and nonreproductive individuals) than males for all scent sources (Figure 1a and 1b, Table 1); the time spent investigating a stimulus did not differ with odor source (Kruskal–Wallis ANOVA on ranks: $H = 3.35$, $df = 3$, $P = 0.35$; Figure 1b). In contrast, males spent different amounts of time investigating each scent type (Kruskal–Wallis ANOVA on ranks: $H = 10.8$, $df = 3$, $P = 0.01$; Figure la). Specifically, post-hoc analyses revealed that males spent more time investigating soiled shavings than secretions of the anogenital area (Dunn's method of pairwise multiple comparison: $q = 5.24$, $P < 0.05$; Figure la).

Experiment 2. When stimuli were presented without a wire mesh, animals sniffed, touched, and licked the samples. As a consequence, the response of test animals varied both in time spent investigating each stimulus and preference for a given stimulus. Males devoted more time to investigating stimuli from reproductive than nonreproductive females for all stimulus sources (Figure 1c, Table 1). Similarly, females spent more time investigating stimuli from reproductive versus nonreproductive males (Figure 1d, Table 1). No differences were detected in the time that each sex spent investigating urine from opposite sex individuals (Figure 1c and d, Table 1). For the three other odor sources females spent more time than males investigating stimuli from opposite sex individuals (Figure 1c and d, Table 1). Females spent similar amounts of time at each source (one-way ANOVA: $F = 0.583$, $df = 41$, $P = 0.63$; Figure 1d). In contrast, the amount of time that males spent investigating each source differed significantly among stimulus types (Kruskal–Wallis ANOVA on ranks: $H = 9.02$, $df = 3$, $P = 0.03$; Figure 1c). Specifically, males spent more time investigating both urine and shavings than they did investigating feces and anogenital secretions (Dunn's method of pairwise multiple comparison, urine vs. feces: $q = 3.45$, $P < 0.05$; urine vs. anogenital area: $q = 3.84$, $P < 0.05$; soiled shavings vs. feces: $q = 3.53$, $P < 0.05$; soiled shavings vs. anogenital area: $q = 4.02$, $P < 0.05$; Figure 1c).

For each stimulus source, responses differed when animals relied on olfaction alone compared to olfaction plus direct interaction with a source. Using olfaction alone, females were able to discriminate the reproductive condition of males only for urine; when direct contact with the stimulus was allowed, females could detect male condition using all four test stimuli. Nonetheless, no differences

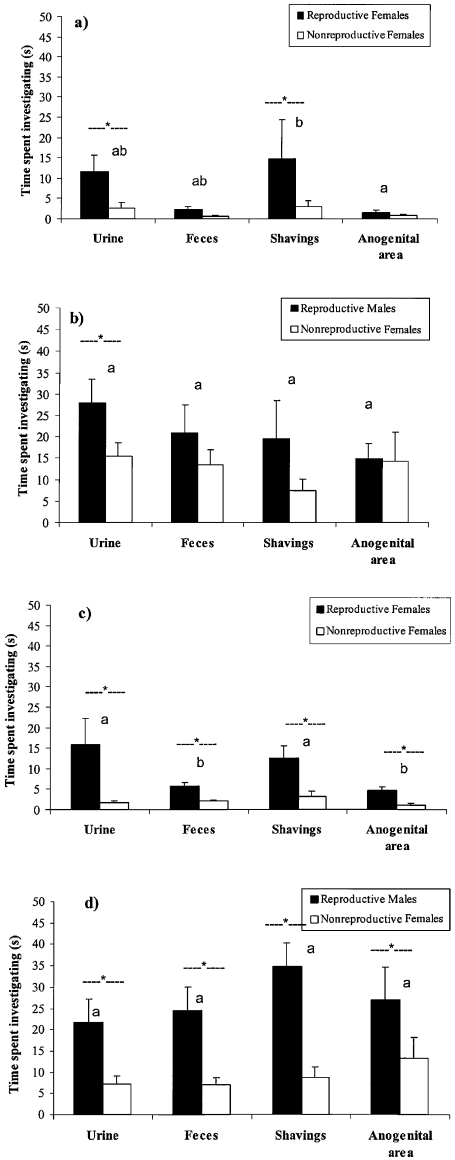

FIG. 1. Length of time (mean ± SE) that reproductive *Ctenomys talarum* individuals investigated stimuli from reproductive and nonreproductive individuals of the opposite sex. Stimuli were from the following sources: urine, feces, soiled shavings, and anogenital area covered with wire mesh in males (a) and females (b), and without wire mesh in males (c) and females (d). *Significant differences between odor pairs. Different letters indicate significant differences in total time animals spent investigating each odor source.

TABLE 1. STATISTICAL PARAMETERS OF THE DURATION OF TIME DEVOTED TO INVESTIGATION BY TEST SUBJECT *Ctenomys talarum* REPRODUCTIVE INDIVIDUALS WHEN EXPOSED SIMULTANEOUSLY TO A PAIR OF ODOR STIMULI. COMPARISONS BETWEEN SEXES FOR TIME SPENT INVESTIGATING EACH ODOR SOURCE ARE ALSO PROVIDED

Odor source	Test animal: Male[a]	Test animal: Female[a]	Male- Female comparison[b]
Experiment # 1			
Odor stimuli: reproductive vs. nonreproductive opposite sex individuals (with wire mesh)			
Urine	$T = 5, n = 10, z = 2.29, P = 0.02$	$t = 2.31, \text{df} = 14, P = 0.04$	$T = 82, n = 10, N = 15, P = 0.008$
Feces	$T = 26, n = 15, z = 1.93, P = 0.05$	$t = 56, n = 15, z = 0.23, P = 0.85$	$T = 142.5, n = 15, N = 15, P < 0.001$
Soiled shavings	$T = 17, n = 10, z = 2.23, P = 0.03$	$T = 16, n = 10, z = 1.17, P = 0.28$	$T = 166, n = 10, N = 14, P = 0.02$
Anogenital secretions	$T = 23, n = 10, z = 0.46, P = 0.65$	$t = 0.14, \text{df} = 12, P = 0.89$	$T = 72, n = 11, N = 13, P < 0.001$
Experiment # 2			
Odor stimuli: reproductive vs. nonreproductive opposite sex individuals (without wire mesh)			
Urine	$T = 0, n = 10, z = 2.80, P = 0.005$	$T = 3, n = 10, z = 2.45, P = 0.012$	$T = 85.5, N = 10, N = 10, P = 0.15$
Feces	$T = 0, n = 10, z = 2.80, P = 0.002$	$T = 1, n = 10, z = 2.70, P = 0.007$	$T = 61, n = 10, N = 10, P = 0.005$
Soiled shavings	$T = 0, n = 10, z = 2.80, P = 0.005$	$T = 0, n = 10, z = 2.80, P = 0.005$	$T = 69, n = 10, N = 10, P = 0.007$
Anogenital secretions	$T = 0, n = 10, z = 2.80, P = 0.005$	$T = 0, n = 12, z = 3.06, P = 0.002$	$T = 82, n = 10, N = 12, P = 0.03$
Experiment # 3			
Odor stimuli: same sex vs. opposite sex reproductive individuals (with wire mesh)			
Urine	$T = 22, n = 10, z = 0.56, P = 0.58$	$T = 0, n = 10, z = 2.80, P = 0.005$	
Feces	$T = 4, n = 10, z = 2.34, P = 0.02$	$T = 0, n = 10, z = 2.80, P = 0.002$	
Soiled shavings	$T = 19, n = 10, z = 0.87, P = 0.39$	$T = 0, n = 10, z = 2.80, P = 0.005$	
Experiment # 4			
Odor stimuli: same population origin vs .different population origin, opposite sex individuals (with wire mesh)			
Urine	$T = 21, n = 12, z = 1.41, P = 0.16$	$T = 41, n = 12, z = 0.16, P = 0.88$	$T = 96.0, n = l2, N = 12, P = 0.02$

[a] Paired *t*-test or Wilcoxon matched-pairs signed-ranks test.
[b] Mann–Whitney test.

were detected in the time devoted to investigating each source under the two experimental conditions (urine: Mann–Whitney: $T = 105, n = 10, N = 15, P = 0.17$; feces: $T = 121, n = 10, N = 15, P = 0.64$; soiled shavings: $T = 127, n = 10, N = 10, P = 0.10$; anogenital area: $T = 159, n = 12, N = 13, P = 0.89$). For males, discrimination of the reproductive condition of females was limited to urine and soiled shavings when only olfaction was allowed but discrimination among all four odor sources was detected when test animals were able to contact the odor source directly. No differences were detected in the time devoted to each source under the two experimental conditions for urine (Mann–Whitney: $T = 112$, $n = 10$, $N = 10$, $P = 0.63$) and soiled shavings ($T = 147$, $n = 10$, $N = 15$, $P = 0.21$). For feces and anogenital secretions, however, the time devoted to investigating samples increased when direct contact with test stimuli was possible (feces: $T = 183.5, n = 10, N = 15, P = 0.003$; anogenital area: $T = 73, n = 10$, $N = 13$, $P = 0.004$).

Experiment 3. Males spent equivalent amounts of time investigating scents from same sex and opposite sex individuals both for urine and soiled shavings (Figure 2a, Table 1). For feces, however, males preferred the odors of other males (Figure 2a, Table 1). Females preferred the odors of males to that of other females for all three odor sources used (Figure 2b, Table 1), suggesting that these olfactory cues provide females with sexually distinct information. Whereas females did not differ in the amount of time spent investigating each odor source (one-way ANOVA: $F = 0.58$, df $= 41$, $P = 0.63$), the amount of time that males spent investigating each source differed significantly among odor types (Kruskal–Wallis: $H = 6.33$, df $= 2$, $P = 0.042$). Specifically, males spent more time investigating soiled shavings than urine (Student–Newman–Keuls method of pairwise multiple comparisons: $q = 3.38$, $P < 0.05$) or feces ($q = 3.96$, $P < 0.05$).

Experiment 4. Males did not distinguish between urine from reproductive females of the same population and that from reproductive individuals from a different population (mean $\pm$ SE: 4.13 ± 0.78 sec and 2.85 ± 0.49 sec for Mar de Cobo and Necochea females, respectively). The same lack of discrimination was shown by females (mean $\pm$ SE: 17.93 ± 6.16 sec and 22.14 ± 7.60 sec for Mar de Cobo and Necochea males, respectively). Females spent more time investigating opposite sex urine than did males (Table 1).

DISCUSSION

This study shows that *C. talaram* individuals discriminate the reproductive condition of opposite sex individuals by olfaction using volatile chemical cues contained in urine (both sexes) and soiled shavings (females). In contrast, when animals could smell, touch, and lick the scent samples, both sexes preferred cues from reproductive individuals regardless of the scent source. Individuals that were

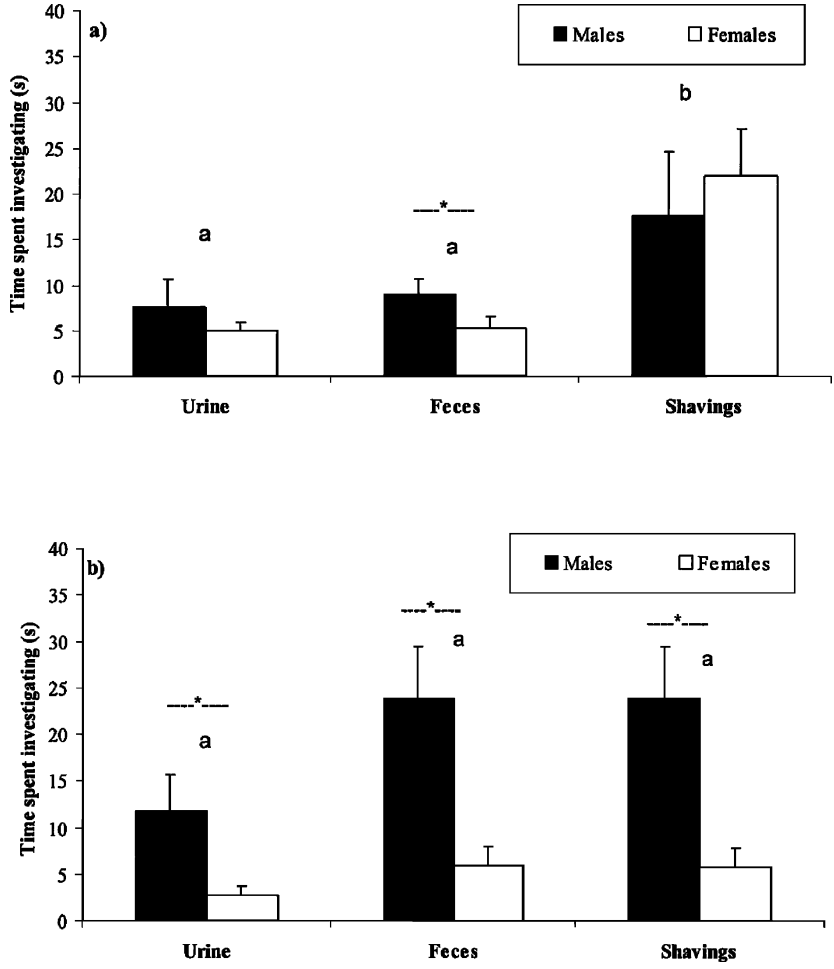

FIG. 2. Length of time (mean ± SE) that reproductive *Ctenomys talarum* males (a) and females (b) investigated reproductive male versus reproductive female odors from the following sources: urine, feces, and soiled shavings. *Significant differences between odor pairs. Different letters indicate significant differences in total time animals spent investigating each odor source.

allowed direct contact with the stimuli tended to spend greater amounts of time investigating the scent sample than when the stimulus was covered with wire mesh. No preferences were detected for opposite sex urine from members of an animal's own versus another population.

While investigating a sample, test animals showed a stereotyped head-bobbing behavior over the scent sample. Although the exact function of this behavior is not known, it has been suggested that head bobbing may facilitate entry of chemical stimuli into the vomeronasal organ (VNO; Wysocki et al., 1980).

In rodents, the olfactory and vomeronasal systems seem to be of primary importance in mediating social recognition (Johnston, 1985; Petrulis et al., 1999). The VNO appears to be specialized to detect nonvolatile molecules, requiring direct contact with the odor stimuli to operate (but see O'Connell and Meredith, 1984). In particular, the VNO appears to be involved in mediating reproductive behaviors whereas the main olfactory system is apparently associated with feeding behaviors or discrimination of social odors such as individual and kin recognition (Wysocki, 1979; Johnston and Rasmussen, 1984; Petrulis et al., 1999).

Chemical signals appear to mediate sexual attraction leading to breeding in many species. The reproductive condition of females may influence the chemical signals they produce, as well as their responses to signals from conspecifics. For example, oestrus female hamsters were more attractive to males (Johnston, 1979; Huck et al., 1989) and displayed stronger preferences for male odors when compared to anoestrus or diestrus females (Johnston, 1980). In meadow voles, which lack a repeated oestrus cycle, a complex, temporally variable pattern of odor production was documented over the reproductive season (Ferkin and Johnston, 1995). In meadow voles, the attractiveness of male odors to females (Ferkin et al., 1994) and, in prairie voles, the amount of time prairie vole females spent with a male during partner preference tests (Klein et al., 1999) were positively related to testosterone titers.

C. talarum females are induced ovulators (Weir, 1974) that undergo two rounds of pregnancy (the latter overlapped with lactation) during the annual breeding season (Malizia and Busch, 1991). Females used in this study were probably at different stages of their reproductive cycle and this could have affected their attractiveness to conspecifics as well as their investigative behavior toward scent stimuli. Although such differences may have introduced variation into observed responses to odors, the results obtained allow us to infer that, like female hamsters (Landauer et al., 1978), the attractive quality of tuco-tucos' odor is not limited to the oestrus period. On the other hand, *C. talarum* males establish dominance hierarchies (Zenuto et al., 2002), and differences in individual levels of aggressiveness may be related to different testosterone titers and also differences in scent signal quality.

C. talarum are polygynous rodents (Zenuto et al., 1999) that do not associate regularly with the opposite sex except for mating; they live alone in burrow systems and are extremely aggressive toward conspecifics. During courtship encounters in captivity, members of a mating pair appear to actively use chemical communication: both sexes sniff burrow entrances where animals usually deposit plant material littered with urine and feces and both sexes mark the walls of artificial

burrow systems with urine and anogenital secretions (Zenuto et al., 2001, 2002). Prior to copulation, individuals engage in mutual sniffing of the anogenital area, each sex marks with urine near the mating partner and then displays head-bobbing over the urine deposited by the opposite sex individual, and both members of a pair engage in self-grooming of the anogenital area. The complex courtship behavior of *C. talarum* includes vocalizations, tactile signals, and chemical cues (Zenuto et al., 2002) that are probably involved in sexual recognition and mate selection, in serving to diminish aggression levels between members of a potential mating pair, and in demonstrating readiness to copulate. Our results suggest that use of chemical signals in these contexts may be improved when direct contact with odor sources is allowed, because the number of odor sources from which reproductive condition could be determined was greater for trials in which animals were allowed to contact odor sources directly versus trials in which detection of reproductive condition was by olfaction alone.

Overall, the response of both sexes of *C. talarum* to odor cues was similar in the breeding and in the nonbreeding season. In both seasons, females typically spent more time investigating male odors than did males investigating female odors. This is in contrast to the results of studies of several other species, in which attraction to sexual odors changed according to breeding season. For example, in the mole-rat *S. ehrenbergi*, both sexes respond differently to urinary chemosensory cues during the breeding and nonbreeding seasons. In the nonbreeding season, when activity is severely reduced, mole rats avoid conspecific urine of both sexes, although such avoidance is not evident during the breeding season. Season-dependent changes in odor preferences in two species of voles (Ferkin and Seamon, 1987; Ferkin, 1990; Ferkin et al., 1995) coincide with varying patterns of social organization. These differences in odor preferences were attributed to seasonal changes in ovarian hormones (Ferkin and Zucker, 1991), although Ferkin and Johnston (1993) found that gonadectomy had differential effects on the attractiveness of different scent sources (feces, mouth, and the posterolateral region). Thus, whereas gonadal hormones play a role in the production of sexually distinctive scents, other factors (e.g., induced vs. spontaneous ovulation; Brown 1985) may also be involved.

C. talarum males are more aggressive than females (Zenuto et al., 2002). As a result, females may be particularly attentive to male odors because males represent both sexual attraction and risk of aggressive interactions. In contrast, males may not pay more attention to the odors of one sex versus the other because individuals of both sexes represent important stimuli (males for aggression and females for reproduction, respectively). Moreover, greater attention to same sex feces by males during the breeding season may be attributed to the high competition for breeding opportunities among males in a polygynous breeding system. The ability to identify sex by olfactory cues has selective advantages in reproduction: it could facilitate mate location and mobilization and increase reproductive

efficiency (August, 1978). Because *C. talarum* are asynchronous in their pattern of activity (Luna et al., 2000), the use of chemical signals may be particularly advantageous for these aggressive subterranean animals since scent deposition in burrow entrances might serve to convey information about the gender of each burrow owner without requiring synchronized activity between signaler and receiver. Encounters in captivity allow us to infer that males are the active mate-seeking sex (Zenuto et al., 2002). In this case it would be advantageous for breeding males to discriminate between male and female burrows before entering another individual's burrow and risking wasted energy or injury if the resident is another male.

Individual *C. talarum* did not show preferences for opposite sex individuals from their own or a different population even though the populations studied differ with regard to karyotype (Mar de Cobo: $2n = 48$, Necochea: $2n = 49$–50; Massarini et al., 1995) as well as numerous behavioral and demographic attributes (Busch et al., 1989; Malizia and Busch, 1991; Malizia et al., 1995). This suggests that there was no differentiation of individuals from the two different populations and that urine does not contain chemical cues that could act as a mechanism of reproductive isolation in this species. However, donor and tested individuals from both populations were maintained under the same diet in the laboratory. Whether differences in grassland composition at the source localities (Comparatore et al., 1991, 1995) can promote natural differences in metabolic odors remains to be evaluated. The odor of urine, feces, and the anogenital area from guinea pigs and meadow voles has been shown to vary with diet and to elicit different investigative behavior from conspecifics (Beauchamp, 1976; Ferkin et al., 1997b). Such differences may be attributed to changes in the quality or quantity of secretions or more likely, to direct influences of protein content on the chemical composition of scents (Ferkin et al., 1997b).

Finally, chemical cues included in metabolic wastes provide *C. talarum* with information about reproductive condition and gender of conspecifics. The mechanisms that mediate such discrimination vary because smelling, touching, and licking of scent samples are important for reproductive condition evaluation whereas only smelling is required for gender recognition. At the same time, however, individuals do not recognize differences in urine from reproductive, opposite sex individuals from different populations. The information provided in this study and the evidence that tuco-tucos are capable of recognizing individual scents (Zenuto and Fanjul, 2002) allow us to infer that chemical cues are important for territorial and breeding performance in this highly territorial subterranean rodent. Nonetheless, several aspects of chemical communication in this species require further clarification, including the reason why females spend more time investigating odor cues than males do, why the investigative behavior of males versus females differs, how behavior (mating preferences, marking behavior, aggressive behavior) is altered as a function of information extracted from scents, and how

hormone level variation affects behavior. Studies of the latter topic in particular would provide critical information regarding the function of biological odors and their underlying physiological bases.

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MALE SEX PHEROMONAL COMPONENTS DERIVED FROM METHYL EUGENOL IN THE HEMOLYMPH OF THE FRUIT FLY *Bactrocera papayae*

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Abstract—Pharmacophagy of methyl eugenol (ME)—a highly potent male attractant, by *Bactrocera papayae* results in the hydroxylation of ME to sex pheromonal components, 2-ally-4,5-dimethoxyphenol (DMP) and (*E*)-coniferyl alcohol (CF). These compounds, which are also male attractants, are then sequestered and stored in the rectal gland prior to their release during courtship at dusk. Chemical analyses of the digestive tract (excluding the crop and rectal gland) showed the absence of the sex pheromonal components and their precursor, ME. However, *B. papayae* males were attracted to and fed on the ME-fed male hemolymph extracts but not on hemolymph extracts of ME-deprived males. After thin layer chromatography in a hexane:ethyl acetate solvent system, flies were attracted to and fed on the original point on the TLC plate where the hemolymph extract had been spotted, suggesting that the pheromone components were bound in polar complexes. Chemical analyses of the ME-fed male hemolymph and crop extracts revealed the presence of the sex pheromonal components. The presence of the ME-derived pheromonal components and the absence of ME in the hemolymph suggest that the hemolymph is involved in the transportation of sex pheromonal components from the crop to the rectal gland.

Key Words—*Bactrocera papayae* pharmacophagy, methyl eugenol, 2-allyl-4,5-dimethoxyphenol, (*E*)-coniferyl alcohol, sex pheromone, sequestration, hemolymph, rectal gland, crop.

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INTRODUCTION

The fruit fly *Bactrocera papayae* (Drew & Hancock) (Diptera: Tephritidae), a sibling species of the Oriental fruit fly, *B. dorsalis* (Hendel) is one of the most destructive fruit fly pests of commercial fruits in the southeast Asian region, particularly Malaysia, Indonesia, and Thailand. Because of the destructive nature of these two congeners, there has been considerable study of their taxonomic status, as both species readily interbreed to produce viable offspring, and both have identical sex pheromonal components derived from methyl eugenol (ME) (Tan, 1993, 2000; Tan and Nishida, 1996, 1998). Recent data suggests that *B. papayae* and *B. dorsalis* may not be distinct genetic species but possibly strains of the same species (Naeole and Haymer, 2003).

Males of *B. papayae* are strongly attracted to and compulsively feed on methyl eugenol (ME). Consumption of ME by males of *B. papayae* has been found to enhance their mating competitiveness (Tan and Nishida, 1996, 1998; Hee and Tan, 1998). A similar phenomenon was also shown to occur with males of *B. dorsalis* (Shelly and Dewire, 1994; Shelly, 1995). In *B.* papayae, male flies convert the ingested ME to other phenylpropanoids, 2-allyl-4,5-dimethoxyphenol (DMP) and (*E*)-coniferyl alcohol (CF) (Nishida et al., 1988; Tan and Nishida, 1996). These metabolites are sequestered in the male rectal gland and subsequently released during courtship at dusk. ME itself has never been detected in the rectal gland of ME-fed males (Nishida et al., 1988; Tan and Nishida, 1996). Behavioral studies have further demonstrated that both DMP and CF function as male sex and aggregation pheromones for *B. papayae* (Hee and Tan, 1998), henceforth referred to as sex pheromonal components.

Whereas the ecological relationship between the attractant, ME and the fruit fly, *B. papayae*, has been shown to involve the production of bioactive derivatives from ME as pheromonal components, little is known about the physiological fate of these bioactive compounds in relation to pheromone communication, especially in the production and transportation of these compounds to the rectal gland where release occurs. Here, we report the presence of the male sex pheromonal components derived from ME in the hemolymph and crop of male *B. papayae*.

METHODS AND MATERIALS

Insects. A colony of *B. papayae* (12 generations per year) was originally collected from infested starfruits, *Averrhoa carambola* L. in 1995, and cultured using an artificial diet as described by Hee and Tan (1998). Adult flies were fed a diet containing yeast, sugar, protein, and water. Males and females were segregated 1–3 d after emergence (DAE). Flies were maintained under ambient conditions in an insectary with a 12L:12D cycle and 83–90% RH at 25–29°C. Sexually mature virgin males (14–20 DAE) that responded maximally to ME (Tan et al., 1987)

were used for experimentation. All experiments on males were performed in the morning during the peak period (0830–1100 hr) of response to ME (Tan, 1985).

Chemicals. ME (purity >98%) was purchased from Merck-Schuchardt, Germany. Authentic standards of 2-allyl-4,5-dimethoxyphenol (DMP) and (*E*)-coniferyl alcohol (CF) (96% *trans*) were synthesized and provided by R. Nishida, Kyoto University, Japan. An emulsion of ME at a concentration of 26.3 mg per 100 μl of 1% Tween® 80 (polyoxyethylene sorbitan monooleate) solution was prepared for feeding male flies.

Consumption of Methyl Eugenol. A male fly was placed with ventral side up and wings held firmly with plasticine. It was directly fed with 0.5 μl (containing 105 μg) of ME using a capillary micropipette. To avoid direct contact with other bodily parts, a small piece of plastic netting was placed across the legs.

Gas Chromatography-Mass Spectrometry (GC-MS). Methods employed in identification of sex pheromonal components were based on those used by Nishida et al. (1988). GC-MS analyses were performed on an HP 5989B mass spectrometer (electron impact, at 70 eV) connected to a non-polar GC column (30 m × 0.25 mm HP-5MS, 0.33-μm film thickness) programmed from 80°C (1-min hold) to 240°C at 10°C/min. Compounds were identified by comparison of retention times and mass spectra with those of authentic standards.

GC quantifications of sex pheromonal components were done on a Shimadzu GC-14A gas chromatograph using an HP Ultra-1 capillary column (25 m × 0.2 mm, 0.33-μm film thickness), with the same temperature program as above, calibrating with authentic standards of known concentrations, using a C-R6A integrator (Shimadzu).

Extraction of Digestive Tract

Gut Tissues. Dissection to remove the digestive tract was performed as described by Hee and Tan (2000). The digestive tract (excluding the crop and rectal gland) from 3 ME-fed males was extracted in Clarke insect saline 30 min after initial feeding on ME; sex pheromonal components were already detectable in the rectal gland within this time (unpublished data). For chemical analyses, 10 μl of ethanol were added to the dissected tissues as solvent, whereas for detection of bioactive samples, 10 μl of Clarke insect saline solution were used instead. One-microliter injection samples were used. A similar procedure was repeated using ME-deprived males as control.

Crop Tissues. The crop of a sexually mature virgin ME-fed male was dissected 5 min after initial feeding with ME and subjected to several rinses of saline, water, and ethanol to remove traces of contamination. The time of crop dissection was chosen because *in vitro*, crop extracts of *B. papayae* male are able to rapidly convert ME to sex pheromonal components within 2–5 min (Lim et al., 1998). This procedure was repeated for another 9 crop organs. A total of 10 crops were

homogenized with 30 μl of saturated NaCl solution and 15 μl of redistilled ethanol. The homogenate was then subjected to thorough mixing and ultrasonication. Then, 20 μl of benzene:ethyl acetate (3:1, v/v) were added and the mixture was thoroughly mixed and centrifuged at 1000 *g* for 5 min. The aqueous phase was re-extracted with 20 μl of benzene:ethyl acetate solution and the combined organic solution was concentrated under a gentle nitrogen flow to approximately 5 μl. One and two microliter portions of the sample were used for GC and GC-MS injections, respectively. As controls, crop samples were extracted in a similar procedure from ME-deprived males.

Hemolymph Extraction. Hemolymph was obtained from sexually mature virgin ME-fed males 30 min after initial feeding on ME through a small longitudinal slit made along the center of the meso- and metathoracic segments, using a disposable glass capillary micropipette with a drawn-out tip. On average, ca. 0.5 μl of hemolymph were collected from each male. Approximately 15–20 μl of hemolymph were accumulated in a 0.2-ml microcentrifuge tube (on ice) containing trace amounts of phenylthiocarbamide (1-phenyl-2-thiourea) to inhibit tyrosinase activity. The combined hemolymph was then centrifuged at 12,000 *g* for 15 min at 4°C. As control in the experiment, the procedure was repeated using ME-deprived males. A new micropipette was used for each set of hemolymph collections to prevent cross contamination. For GC and GC-MS analyses, 2 μl of the hemolymph were added to 10 μl of ethanol and 1-μl aliquots of the mixture were used for GC-MS and GC injections.

Attraction of Male B. papayae to Extracts of Digestive Tract and Hemolymph of ME-Fed Conspecific Males. To ascertain whether the digestive tract (excluding the crop and rectal gland) and/or hemolymph contained the sex pheromonal components, a simple bioassay was devised. The bioassay worked on the principle that sexually mature males of *B. papayae* are attracted to the sex pheromonal components at quantities often undetectable by GC (Nishida et al., 1988; Tan, 2000). Therefore, attraction of the males to and feeding on the digestive tract and/or hemolymph extracts would indicate the possible presence of the pheromonal components before subjecting the positive extract for further chemical analyses. Male flies used in this method had not been exposed to DMP or CF, because exposure may induce habituation to the pheromonal compounds (Khoo et al., 2000). Two microliters each of the ME-fed male digestive tract and hemolymph extracts were separately dispensed as two spots on a Whatman® No. 1 filter paper (diam 11.0 cm) using Drummond® micropipettes (32 mm). The filter paper was placed in a cage (43 × 43 × 43 cm) containing 100 mature males of *B. papayae* for 15 min. The attraction and behavior of the flies were observed. Extracts attractive to males were then subjected to thin layer chromatographic analyses. In the control experiment, the procedure was repeated using extracts from ME-deprived males. Three replicates were conducted using different batches of males to avoid habituation to the volatiles.

Thin Layer Chromatography (TLC) and Assay of Bioactive Spots. Portions of the bioactive extracts (10 μl) previously identified as attractive to flies were subjected to TLC on a precoated plate (HPTLC, silica gel 60 F_{254}, nano TLC, Merck) and developed with hexane:ethyl acetate (2:1, v/v) prior to visualization under UV at 254 nm. As controls, authentic DMP and CF (each 1 μl of 10 mg/ml solution) were spotted separately on different lanes. All samples were allowed to dry completely before being developed. Spots indicating DMP and CF were marked with pencil and covered with a small piece of 3M® Scotch™ tape to prevent attraction of males in the subsequent detection of active spots. The developed plate then was slightly moistened by misting with deionized water to enhance the evaporation rate of volatiles (Hee and Tan, 1998). The plate was introduced into a cage (43 cm × 43 cm × 43 cm) containing 100 sexually mature flies. Their attraction and behavior were observed for 15 min to determine the active zones. Three replicates were performed each time using different batches of flies.

Separation of Hemolymph Extracts in the Original Spot on the TLC Plate. Following development on the TLC plate containing ME-fed male hemolymph with hexane:ethyl acetate (2:1, v/v), the original spot on the TLC plate (found previously to be attractive to conspecific males) was allowed to dry completely. Then, the original spot was scraped into a glass tube (Tan, 1972). One ml of ethanol was added to the silica gel in the tube and the mixture was thoroughly stirred, then centrifuged at 3000 *g* for 10 min. The resulting supernatant was transferred to a new tube, and 1 ml of ethanol was added to the pellet. This procedure was repeated twice. The accumulated supernatant was then concentrated further under partial pressure (Buchi® rotary evaporator) to near dryness. The concentrated sample was resolubilized in 30 μl of ethanol for GC-MS and GC analyses, injecting 1-μl aliquots. As controls, ME-deprived male hemolymph was subjected to the procedure. The experiment was performed in triplicate.

RESULTS

Male Attraction to Extracts of Digestive Tract and Hemolymph of ME-Fed Males. Extracts of the digestive tract (excluding the crop and rectal gland) of ME-fed male *B. papayae* flies when presented to sexually mature males did not elicit any attraction or feeding activity. Subsequently, ME and sex pheromonal components were not detected in analyses of the digestive tract extracts. However, male flies were attracted to the spot on the TLC plate containing hemolymph from ME-fed males, exhibiting rapid short zig-zag flight before landing and feeding on the hemolymph spot. In contrast, there was no attraction to hemolymph extract of ME-deprived males (Figure 1).

Thin Layer Chromatography (TLC) and Biodetection. The developed TLC plate containing extracts of hemolymph from ME-fed males did not show the presence of pheromonal components when viewed under UV illumination at 254 nm

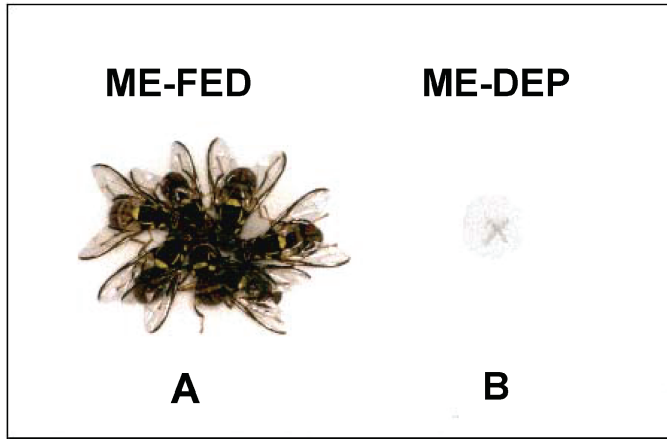

FIG. 1. Attraction of sexually mature virgin males of *Bactrocera papayae* to hemolymph extracts. (A) Males feeding on spot containing hemolymph extracts of methyl eugenol-fed (ME-FED) males. (B) No attraction of males to spot marked "X" containing hemolymph extracts of methyl eugenol-deprived (ME-DEP) males.

(Figure 2A). However, when the plate was exposed to mature male flies, attraction to and feeding on the original spot were observed as compared with no attraction to the adjacent spot containing hemolymph extract from ME-deprived males (Figure 2B). To discount the possibility that the original spot was overwhelming the attraction of males to other areas on the TLC plate, the original spot was then covered with a piece of scotch tape. Following further exposure of the plate to male flies, little (1–2 males) or no attraction of males to the spots with R_f values corresponding to DMP and CF on the plate was observed.

Gas Chromatographic Analyses. GC analyses confirmed the presence of the sex pheromonal components in the hemolymph extract of ME-fed males (Figure 3). Pheromonal components were identified from matches in retention time and mass spectra with authentic standards of DMP and CF. ME was not found.

The sex pheromonal components and ME were also detected in the crop extract of ME-fed males (Figure 3B). In addition, after TLC, DMP and CF were also found in the ME-fed male hemolymph extract, both in the original spot and in the spots having similar R_f value to DMP ($R_f = 0.45$) and CF ($R_f = 0.26$), respectively (Table 1).

DISCUSSION

ME is found in at least 200 species of plants from 32 families, and has been shown to play a central role in the chemical ecology of ME-sensitive fruit

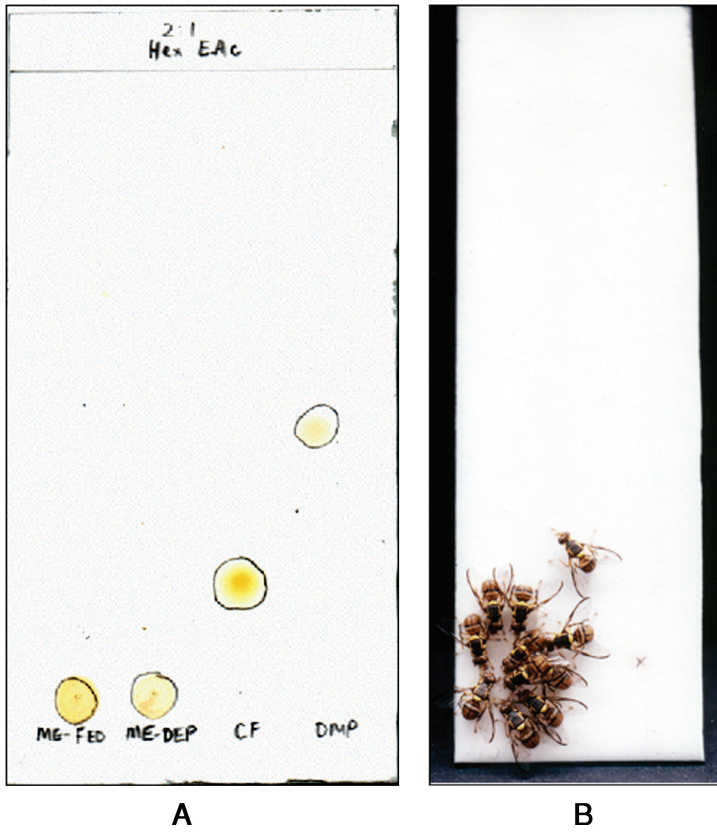

FIG. 2. (A) Thin layer chromatogram of hemolymph spots from methyl eugenol-fed (ME-FED) and -deprived (ME-DEP) males. Separated spots of authentic standards of CF and DMP are shown on the right. Note that CF and DMP were not detected in hemolymph extracts of ME-fed and -deprived males. (B) Attracted males feeding on original spot containing the methyl eugenol-fed male hemolymph (left) and no attraction to spot marked "X" containing hemolymph of methyl eugenol-deprived male (right) following thin layer chromatography.

fly species (Tan, 1993, 2000; Tan and Nishida, 1998). Consumption of ME and its conversion to DMP and CF in ME-fed male flies has been shown to confer protection against vertebrate predators (Nishida and Fukami, 1990; Tan and Nishida, 1998; Wee and Tan, 2001). Further, ME and DMP have also been detected from *Bulbophyllum* orchids such as *Bu. cheiri* (Tan et al., 2002; Nishida et al., 2004). In *Bu. vinaceum*, both DMP and CF were detected along with ME

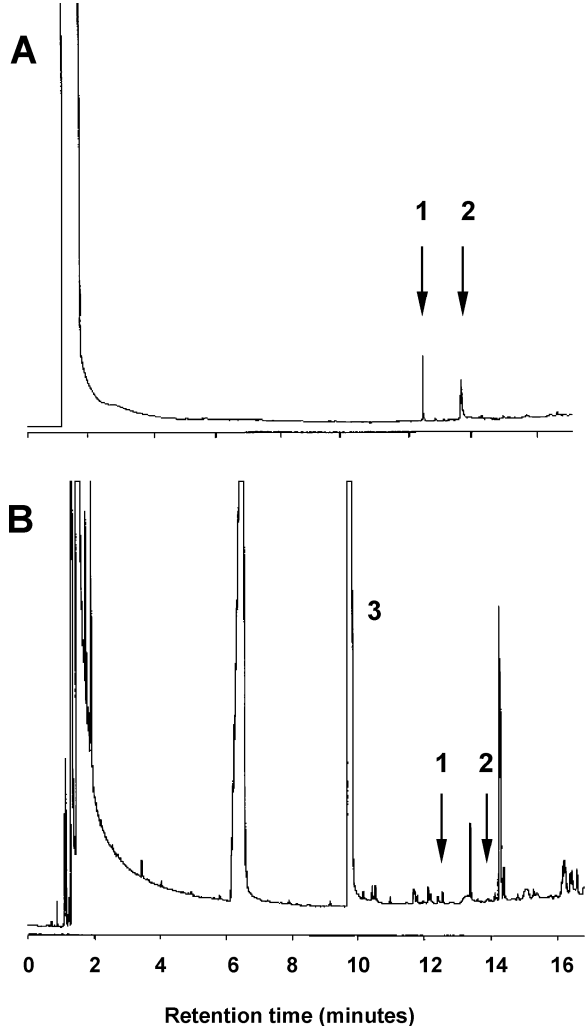

FIG. 3. Gas chromatograms of sex pheromone components present in (A) hemolymph and (B) crop extract (in trace amounts) of methyl eugenol-fed *Bactrocera papayae* males. **(1)** **2**-allyl-4,5-dimethoxyphenol, **(2)** (*E*)-coniferyl alcohol, and **(3)** methyl eugenol.

as major components in floral fragrance (Tan, K. H. and Nishida, R., unpublished data). The interrelationship and coevolution of this genus of wild orchids and ME-sensitive *Bactrocera* species are currently being examined (Tan and Nishida, 2000; Tan et al., 2002).

TABLE 1. SEX PHEROMONE COMPONENTS[a] RECOVERED FROM HEMOLYMPH[b] OF METHYL EUGENOL-FED *Bactrocera papayae* MALES

	Content (ng)*	
	DMP	CF
Original spot	78 ± 26	87 ± 26
Spot corresponding to DMP ($R_f = 0.45$)	17 ± 14	—
Spot corresponding to CF ($R_f = 0.26$)	—	13 ± 8

[a]After thin layer chromatography (HPTLC, silica gel 60 F_{254}, nano TLC, Merck) using a solvent system of hexane:ethyl acetate (2:1, v/v).
[b]10 μl of hemolymph was dispensed on the original spot.
*Means ± SE, $N = 3$.

This investigation marks the first time that ME metabolites, which function as sex pheromonal components, were detected in both the hemolymph and crop organ of *B. papayae* males following consumption of ME. Further studies (unpublished data) have also suggested that hemolymph transports DMP and CF, as indicated by the presence of possible peptide carriers in hemolymph fractions containing pheromone components. These results suggest that the sex pheromonal components are biosynthesized from a common precursor, ME, in the crop and transported probably by the hemolymph carrier(s) to be sequestered by the male rectal gland. Further light microscope studies have demonstrated the accumulation of oil droplets and autofluorescent compounds in the rectal papillae and sacs of ME-fed males (Khoo and Tan, personal communication). If these oils contain the pheromone components, then the data suggest that the pheromones are transported by hemolymph carriers to the rectal gland via the rectal papillae.

In contrast, endogenously produced terpenoid sex pheromonal components in the hemolymph, crop, and oral secretions of the male Caribbean fruit fly, *Anastrepha suspensa* (Loew) were isolated and identified (Teal et al., 1999; Lu and Teal, 2001). The principal storage/release sites of these terpenoids were the salivary glands, hindgut, anal pouch, and the pleural abdominal glands, which are exposed during courtship displays (Nation, 1990). In addition, the hemolymph of *A. suspensa* has also been suggested to play a role in the transport of these terpenoid sex pheromonal components during sexual signalling (Teal et al., 1999).

Bioassays using *B. papayae* males to detect the presence of ME-derived sex pheromonal components in extracts of the digestive tract (excluding the crop and rectal gland) and hemolymph of ME-fed males were shown to be effective, sensitive, reliable, and economical. This technique enables preliminary screening for the presence of pheromonal components prior to chemical analyses, that are

often tedious to perform and sometimes less sensitive. Although females are known to be attracted to the sex pheromonal components (Nishida et al., 1988; Tan and Nishida, 1996, 1998; Khoo et al., 2000), female attraction has been difficult to observe because when a female is attracted, the fly would be moving, rapidly at times, near the attractant spot. Furthermore, at low doses, females were not observed to extrude their ovipositors (Tan and Nishida, 1996, 1998). Thus, it is difficult to pinpoint the exact spot that female flies were attracted to. In contrast, the use of male flies facilitates the detection of bioactive hemolymph samples because males feed on the spot containing the pheromonal components and leave a distinct salivary mark.

In addition, males have a wider temporal response window than females. This is attributed to the fact that the period during which males are most sensitive to the pheromonal components occurs between 0800 and 1100 hr (Tan, 1985; Nishida et al., 1988; Tan and Nishida, 1996, 1998; Khoo et al., 2000; Wee et al., 2002), whereas that of females is only half an hour, between 1845 and 1915 hr (Hee and Tan, 1998; Khoo et al., 2000).

The behavioral pattern of *B. papayae* males attracted to the extracts of hemolymph of ME-fed males is similar to that of ME, i.e., a sequence of zig-zag anemotaxis toward the source of ME and pulsation of the mouthparts followed by compulsive feeding on ME (Metcalf et al., 1975). DMP and CF are also highly attractive to male flies and elicit a searching behavior similar to that of males to ME (Nishida et al., 1988; Tan and Nishida, 1998; Tan, 2000). Recent probit analysis of *B. papayae* male quantum response to ME showed ED_{50} (effective median dose required to elicit response in 50% of the fruit fly population tested) of 318 ng (Wee et al., 2002). If ED_{50} of DMP and CF are assumed to be within ME range, the low quantity of DMP and CF in the corresponding spots on TLC (having similar R_f values of authentic samples) would attract none or 1–2 males, as evidenced from these results.

Results from the biodetection of bioactive spots following TLC suggest that the solvent system of hexane:ethyl acetate (2:1; v/v) was not able to effectively separate DMP and CF from hemolymph of ME-fed males. In contrast, spot applications of authentic DMP and CF on the TLC plate and the subsequent chromatographic development resulted in these samples being separated easily, and the developed spots were detected under ultraviolet illumination. To discount the possibility that water soluble materials from the hemolymph were interfering with the free movement of solvent through the spot, the samples were allowed to dry completely prior to solvent development. Therefore, the larger amounts of the pheromonal components in the original spot suggest the presence of a higher degree of bound pheromonal components than that of free or unbound components in the hemolymph. As such, the solvent partitioning effect of TLC was not able to dissociate the bound pheromonal components. This suggests the occurrence of a sex pheromone binding complex in the hemolymph of ME-fed males.

Detection of sex pheromonal components in the crop extracts of ME-fed male flies also suggested that it is the site of ME hydroxylation to the ME metabolites, DMP and CF. *In vitro* studies on crop extracts of ME-fed male *B. papayae* have shown the presence of an enzyme with mixed-function monooxygenase activity based on the cytochrome P-450 system (Lim et al., 1998). In addition, the absence of ME in the hemolymph and digestive tract (excluding the crop) suggests that ME is completely converted into DMP and CF in the crop, or the crop membrane is impermeable to ME but ME-derived pheromonal components are probably actively transported across the crop membrane into the hemolymph.

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CUTICULAR HYDROCARBONS OF *Polistes dominulus* AS A BIOGEOGRAPHIC TOOL: A STUDY OF POPULATIONS FROM THE TUSCAN ARCHIPELAGO AND SURROUNDING AREAS

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Abstract—In social insects, the types and proportions of epicuticular lipids may exhibit significant diversity as a result of factors such as age, sex, caste, rank, nest, and relatedness. It is known that these variations can be used by social insects to acquire information regarding conspecific individuals. Recent findings have shown that different populations of *Polistes dominulus* (Christ.) have distinctly different chemical cuticular profiles, and that wasps are able to recognize individuals of their own population. In this study, we showed that cuticular hydrocarbon patterns of *Polistes dominulus* are consistent with similarities among northern Tyrrhenian islands, as reported in previous biogeographic studies. Indeed, our findings indicate that cuticular hydrocarbon mixtures of *P. dominulus* from Capraia and Corsica are grouped together by cluster analysis, while those from Elba and Giglio cluster with cuticular profiles of the mainland wasps (Venturina).

Key Words—Cuticular hydrocarbons, Corsica, Elba, Giglio, Capraia, Tuscany, biogeography, gas chromatography, mass spectrometry, *Polistes dominulus*.

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INTRODUCTION

In insects, epicuticular lipids are important regulators of cuticular permeability, and play an important role in communication. The compounds on the cuticle are mainly long-chained hydrocarbons such as linear alkanes, alkenes, and mono-, di-, and tri-methyl branched alkanes (Howard, 1993). Because cuticular signatures are usually formed by a diverse set of compounds that differ quantitatively, and occasionally qualitatively, among individuals, each signature contains a significant amount of information. Consequently, within a species, epicuticular lipid mixtures may exhibit diversity with respect to age, sex, geographic location, and, in social insects (ants, bees, wasps, and termites), caste, rank, and nest (Howard, 1993; Chapman et al., 1995; Clément and Bagnères, 1998; Singer et al., 1998; Sledge et al., 2001). This diversity is generally linked to variations in the proportion of methyl-branched alkanes and alkenes, rather than to linear alkanes (Nelson, 1993; Chapman et al., 1995; Clément and Bagnères, 1998; Haverty et al., 2000; Sledge et al., 2001).

In nonsocial insects these differences in signatures are used in mate recognition (Howard, 1993), while in social insects, cuticular hydrocarbons are involved in other recognition processes such as nestmate, caste, and rank (Bonavita-Cougourdan et al., 1987; Lorenzi et al., 1996; Dani et al., 2001).

In the honeybee (*Apis mellifera*) and the European paper wasp (*Polistes dominulus*), experimental manipulation of cuticular signatures by chemical supplementation have shown that unsaturated and methyl-branched compounds are more likely to be involved in nestmate recognition than other compounds (Breed, 1998; Dani et al., 2001). Moreover, epicuticular hydrocarbon profiles of *P. dominulus* can vary with respect to province; individuals from neighboring localities have more similar epicuticular profiles than those from more distant ones (Dapporto et al., 2004a).

Here, we compared cuticular hydrocarbons of *P. dominulus* collected from Corsica, the Tuscany coast, and from three islands of the Tuscan Archipelago (Capraia, Elba, and Giglio). The Tuscan Archipelago is comprised of seven main islands (Gorgona, Capraia, Elba, Pianosa, Montecristo, Giglio, and Giannutri) and is situated between Corsica and the Italian Peninsula (Figure 1). The disjunction and rotation of the Corsica–Sardinia microplate from the Iberian Peninsula (Edel et al., 2001) and the biogeographic consequences have been well documented (Dallai, 1983; La Greca, 1983; Caccone et al., 1994). This process began 21 million years (Myr) ago leading to a high proportion of endemism (Bigot, 1958; Médail and Verlaque, 1997). There is evidence that Corsica and Sardinia share some biotic characteristics with the Tuscan Archipelago and, particularly, with Capraia and Montecristo. The floristic associations divided the islands into two blocks: the first includes Corsica, Sardinia, Capraia, and Montecristo and the second includes Elba, Pianosa, Giglio, and Giannutri along with the Italian Peninsula

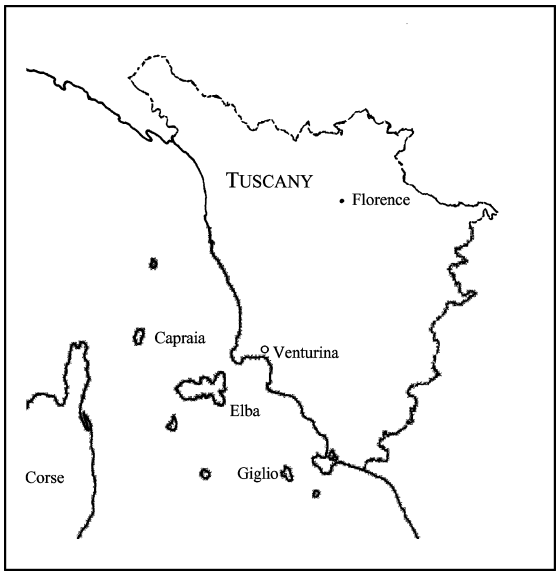

FIG. 1. The Tuscan Archipelago and the surrounding areas.

(Arrigoni, 1975). Studies on faunistic associations showed similar findings for some groups of invertebrates (Lepidoptera: Jutzeler et al., 1996; Dapporto et al., 2003; Hymenoptera: Strumia, 2003).

In this research, we assessed whether variations of the cuticular hydrocarbons of *P. dominulus* coming from Corsica, Tuscan Archipelago (Capraia, Giglio, and Elba), and Italian Peninsula reflect the same biogeographic relationships already highlighted by floristic, faunistic, and biometric approaches (Arrigoni, 1975; Jutzeler et al., 1996; Dapporto et al., 2003; Strumia, 2003). Moreover, we identify the hydrocarbon classes that make it possible to distinguish wasps originating from different areas.

METHODS AND MATERIALS

Insect Species. P. dominulus is a social paper wasp. Its native areas are Mediterranean regions to China. Recently, it has been introduced into Australia and the U.S.A. In spring, fertilized females leave their winter refuges and establish new nests. The first offspring emerge in June and consist of typically sterile females (workers) that assume the duties of foraging (usually nectar and insect larvae), nest construction, and colony defense. In the course of the summer, future female

and male reproductives appear, and mating occurs outside the nests. In autumn, colonies decline, and fertilized females overwinter under rocks or in crevices (Pardi, 1996).

Collection of Cuticular Hydrocarbons. We collected the cuticular hydrocarbons (CHCs) from 20 wasps of five different localities, all at sea level and within 2 km of the coast in Corsica Island (La Marana, $N = 5$), Elba Island (Procchio, $N = 4$), Giglio Island (Castello, $N = 4$), Capraia Island (Isola, $N = 4$), and Tuscany (Venturina, $N = 3$) (Figure 1). All localities share similar environmental characteristics (Mediterranean bushes).

In order to limit the influence of parameters such as colony, sex, rank, and caste, we collected CHCs from flying females from the second half of June to the end of July 2003. It is not easy to distinguish foundresses from workers, but catching flying females in this restricted period ensures a high probability of collecting only workers.

We sampled CHCs using a method similar to that described by Turillazzi et al. (1998). A piece of filter paper was gently rubbed on the wasp's thoracic scutum for 30 sec using sterile forceps. Thereafter, the filter paper was placed in an aluminum sheet.

Chemical Analysis. The epicuticular compounds were extracted from the filter paper in 300 μl of pentane for 10 min. The solution was dried in a nitrogen stream and re-eluted in 25 μl of heptane for GC-MS analysis. We injected 2 μl of solution into an Hewlett Packard (Palo Alto, CA) 5890A gas chromatograph coupled with an HP 5971A mass selective detector. A fused silica capillary column coated with 5% diphenyl-95% dimethyl polysiloxane (Rtx-5MS, 30 m $\times$ 0.25 mm $\times$ 0.5 μm; Restek, Bellefonte, PA) was used in the GC analysis. The injector port and transfer line were set at 280°C, and the carrier gas was helium (at 12 psi). The temperature protocol was: 70–150°C at a rate of 30°C/min (held for 5 min), and 150–310°C at 5°C/min (held for 11.3 min). Analyses were performed in splitless mode. Identification of cuticular compounds was performed on the basis of their mass spectra produced by electron impact ionization (70 eV).

Statistical Analysis. Peak areas of the epicuticular gas chromatogram of each wasp were transformed into percentages of the total CHCs. To visualize the pattern of proximity between wasp chemical profiles we employed squared Euclidian distances (Z-scores were used to standardize the percentages), thus obtaining a dissimilarity matrix, which was subjected to a cluster analysis (Ward's method).

To understand which compounds were responsible for locality differences, we tested for them using the Mann-Whitney U test. All analyses were two tailed, and the level of significance was set at 5%. Conventional P-values are marked with an asterisk to indicate significance ($P < 0.05$), a double asterisk ($P < 0.01$), and a triple asterisk ($P < 0.001$). We employed exact tests as suggested by Mundry and

Fisher (1998). Statistical analyses were performed with Microsoft Excel 5.1 and SPSS 9.05.

RESULTS

We identified 50 hydrocarbons (chain length ranging from 25 to 34 carbon atoms) from the cuticles of 20 wasps. The cuticular chemical mixture was predominantly composed of a series of linear, monomethyl-branched, or dimethyl-branched, saturated hydrocarbons and a low percentage of linear, unsaturated hydrocarbons. Most compounds were linear and monomethyl branched alkanes with an odd-number carbon chain. Table 1 shows median percentages of the chemical compounds that were collected from the wasps of the five localities.

Cluster analysis, performed on the percentages of compound peak areas, separated chemical profiles into two distinct blocks. The first included Corsica (Co) and Capraia (Ca) cuticular signatures, and the second those from Elba (El), Giglio (Gi), and Venturina (Ve). Moreover, within each block, individuals from the same locality did not cluster together, and, consequently, no well-defined subgroups emerged (Figure 2).

We assessed differences in the percentages of each compound between the two distinct biogeographic groups (Co + Ca, $N = 9$ vs. El + Gi + Ve, $N = 11$) in order to understand which compounds were responsible for separation of the two groups. We found significant differences in 48.0% of the cases (see Table 1). Only two of the 10 *n*-alkanes (*n*-C25 and *n*-C29) differed significantly between the two biogeographic groups (Figure 3), while *n*-alkenes showed no significant differences. The most striking dissimilarity was found in methyl- and dimethyl-branched alkanes, which differed in 56.5% and 69.2% of the cases, respectively (Figures 4 and 5).

DISCUSSION

Cuticular hydrocarbon mixtures of *P. dominulus* from Capraia and Corsica grouped together by cluster analysis, while those from Elba and Giglio clustered with cuticular profiles of the mainland wasps (Venturina). This result reflects the pattern of similarity seen in previous biogeographic studies that reported a similar species pattern and subspecific variation within the same two blocks (Arrigoni, 1975; Jutzeler et al., 1996; Dapporto et al., 2003; Strumia, 2003). Some authors (Arrigoni, 1975; Jutzeler et al., 1996; Strumia, 2003) attribute the species distribution pattern in the Tuscan Archipelago to paleogeographic events that occurred in the Northern Tyrrhenian area.

It is known that several of the islands within the Tuscan Archipelago (e.g., Capraia, Gorgona, Montecristo, Giglio, and Elba) were connected to the Italian mainland, and Elba, and maybe Giglio were linked to Tuscany also during the

TABLE 1. MEDIAN PERCENTAGES OF HYDROCARBONS FOR EACH LOCALITY

		Co+Ca ($n = 9$)		El+Gi+Ve ($n = 11$)			
	Chemical compounds	Corsica	Capraia	Elba	Giglio	Venturina	$P_{Ca+Co\ vs.\ El+Gi+Ve}$
1	*n*-C25	4.97	7.68	3.29	4.04	3.88	*
2	11-meC25	1.25	3.30	0.23	0.39	0.27	***
3	7-meC25	0.66	0.59	0.16	0.00	0.00	***
4	3-meC25	0.00	0.00	0.00	0.00	0.00	ns
5	*n*-C26	2.72	4.32	3.43	3.56	3.71	ns
6	11,12,13-meC26	0.74	1.11	0.00	0.00	0.11	***
7	7,11-dimeC26	0.00	0.00	1.06	0.61	0.21	***
8	2-meC26	0.80	1.48	0.54	0.71	0.34	*
9	*n*-C27:1	0.52	0.50	0.55	0.54	0.54	ns
10	*n*-C27	15.31	14.11	14.97	15.01	12.67	ns
11	13-meC27	12.72	15.52	2.76	4.64	3.12	***
12	7-meC27	1.90	0.85	0.46	0.35	0.28	***
13	5-meC27	0.62	0.36	0.36	0.25	0.35	ns
14	11,15-dimeC27	1.27	1.69	0.77	0.57	0.48	***
15	7,*y*-dime+3-meC27	1.17	1.28	2.31	1.84	2.13	ns
16	5,*y*-dimeC27	0.00	0.11	0.00	0.00	0.00	ns
17	*n*-C28	3.78	4.10	6.03	5.10	6.76	ns
18	12,13,14-meC28	0.87	0.76	0.91	0.91	0.72	ns
19	2-meC28	0.91	0.80	1.53	1.58	1.35	ns
20	*n*-C29:1	0.00	0.00	0.00	0.75	1.20	ns
21	*n*-C29	4.75	4.51	10.93	7.27	9.52	**
22	11,13-meC29	11.90	8.06	10.29	10.53	10.17	ns
23	7-meC29	1.82	1.04	0.39	0.41	0.71	**
24	5-meC29	0.60	0.44	0.55	0.53	0.83	ns
25	11,*y*+9,y-dimeC29	0.88	1.11	3.40	2.27	2.31	*
26	7,*y*-dimeC29	0.72	0.54	0.00	0.00	0.29	***
27	3-meC29	0.63	0.44	2.88	2.48	2.45	***
28	5,*y*-dimeC29	0.58	0.32	0.00	0.09	0.49	*
29	*n*-C30	2.36	2.95	3.81	3.61	4.24	ns
30	13,14,15-meC30	0.86	0.49	1.12	1.17	0.92	*
31	2-meC30	0.00	0.29	0.62	0.54	0.51	**
32	*n*-C31:1	0.00	0.11	0.07	0.54	0.25	ns
33	*n*-C31	2.08	2.16	4.07	2.86	3.44	ns
34	11,13,15-meC31	5.83	3.58	7.89	7.47	8.81	*
35	7-meC31	0.50	0.27	0.00	0.00	0.00	***
36	5-meC31	0.60	0.28	0.92	0.76	0.41	ns
37	11,*y*-dimeC31	0.00	0.71	3.68	4.09	5.05	***
38	7,*y*-dimeC31	1.82	0.99	0.00	0.00	0.00	***
39	3-meC31	0.00	0.00	0.74	0.64	0.00	*
40	5,*y*-dimeC31	1.47	0.78	0.18	0.00	0.66	**
41	*n*-C32	1.13	1.45	1.50	1.80	2.21	ns
42	14,15,16-meC32	0.00	0.00	0.00	0.68	0.40	ns
43	*n*-C33:1	0.00	0.00	0.35	0.00	0.00	ns
44	*n*-C33	0.74	1.03	1.19	0.97	1.32	ns
45	11,13,15-meC33	1.85	1.17	2.41	3.07	2.36	ns

TABLE 1. CONTINUED

		Co+Ca ($n = 9$)		El+Gi+Ve ($n = 11$)			
	Chemical compounds	Corsica	Capraia	Elba	Giglio	Venturina	$P_{Ca+Co\ vs.\ El+Gi+Ve}$
46	5-meC33	0.00	0.00	0.00	0.00	0.00	ns
47	11,*y*-dimeC33	0.71	0.91	3.06	3.75	3.21	**
48	7,*y*-dimeC33	0.00	0.00	0.00	0.00	0.00	ns
49	5,*y*-dimeC33	0.63	0.17	0.00	0.00	0.53	ns
50	*n*-C34	0.44	0.62	0.68	0.64	1.07	ns

Note. Mann-Whitney *U*-test results ($P_{Ca+Co\ vs.\ El+Gi+Ve}$) on the differences between the two biogeographic groups are reported. "*y*" is used to indicate the position of the second methyl in the compound was not understandable.
$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

recent Pleistocene glaciations. In contrast, Capraia and Montecristo from the Late Tortonian have not been in contact with the Italian peninsula (Bossio et al., 2000), and geological data are not consistent with a connection between NNE Corsica and Capraia islands. Apart from a deep channel (about 400 m in depth) separating these islands, their magmatic products belong to different phases of

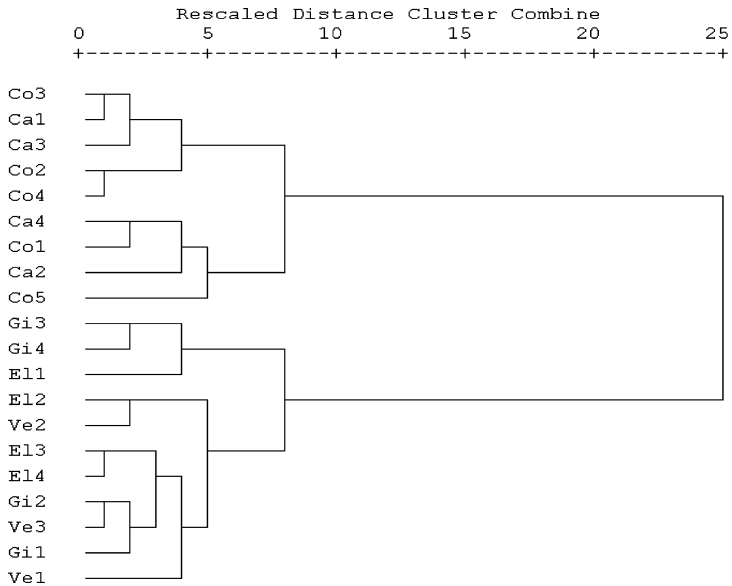

FIG. 2. Cluster analysis (Ward's method) derived from proportions of cuticular hydrocarbons of 20 *Polistes dominulus* workers from Capraia (Ca), Corsica (Co), Giglio (Gi), Elba (El), Venturina (Ve).

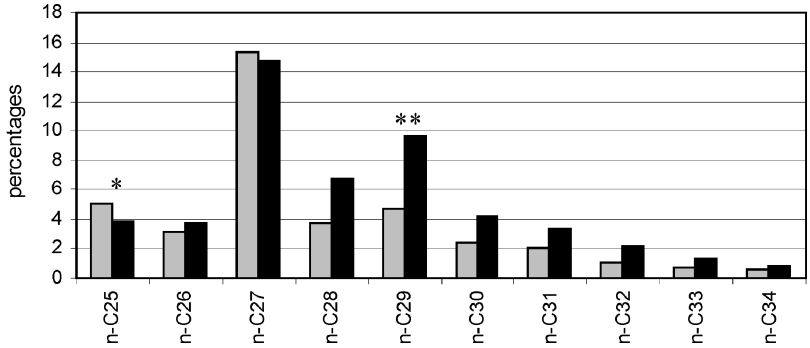

FIG. 3. Median percentages of linear alkanes in the two biogeographic groups (Ca + Co, grey bars; El + Gi + Ve, black bars).

the northern Apennine magmatism and are separated both in space and time. Sisco lamproitic sill (NNE Corsica) range from 15.0 to 13.5 Myr, whereas the activity of Capraia composite volcano is referable to two distinct events ranging from 6.9 to 6.0 Myr and 4.6 to 3.5 Myr, respectively. Moreover, Sisco lamproitc sill and Capraia volcanic rocks show very different chemical, geochemical, and petrological characteristics (Innocenti et al., 1992; Dini et al., 2002).

There is firm evidence that many flying insects are able to cross extensive sea-barriers (Baker, 1984; Benton, 1995). For example, in the Aegean Archipelago, Dennis et al. (2000) showed that species richness, frequency, rarity, and incidence

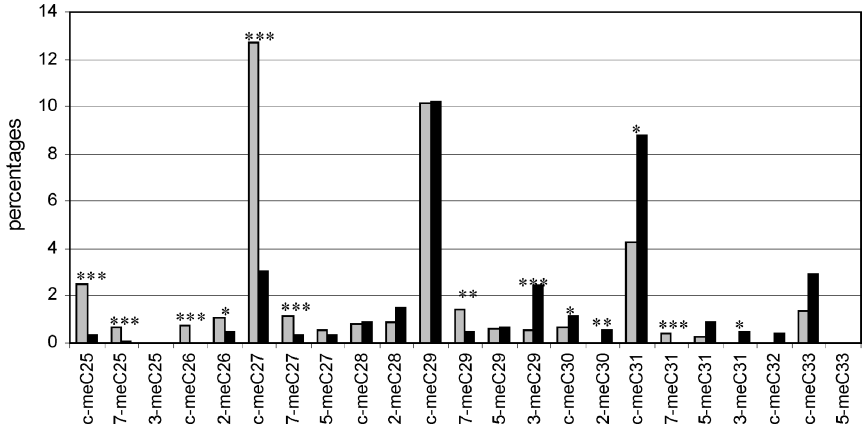

FIG. 4. Median percentages of monomethyl-branched alkanes in the two biogeographic groups (Ca + Co, grey bars; El + Gi + Ve, black bars). "C-me" refers to monomethyl brunches in the middle of chains.

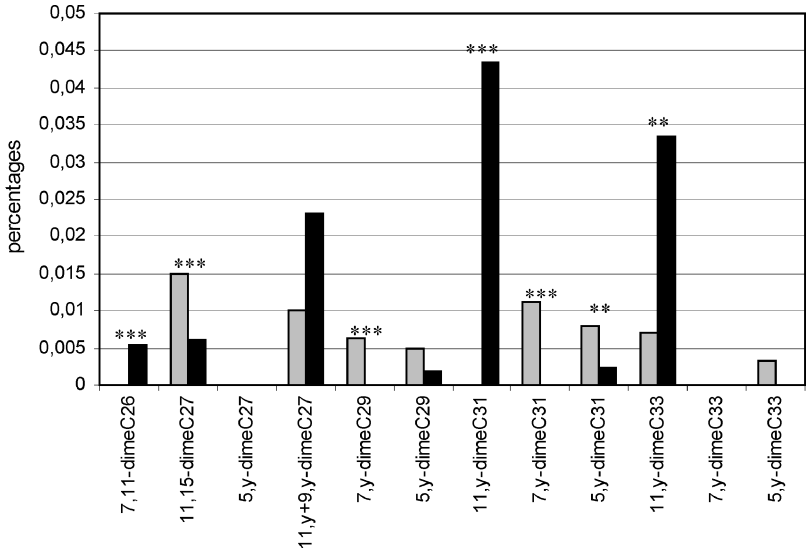

FIG. 5. Median percentages of dimethyl-branched alkanes in the two biogeographic groups (Ca + Co, grey bars; El + Gi + Ve, black bars). "*y*" is used to indicate the position of the second methyl in the compound was not understandable.

of butterflies (one of the most dispersive groups) were mainly affected by contemporary geography rather than paleogeography. In contrast, with less dispersive tenebrionid beetles of the Aegean Islands, paleogeography is responsible for the present level of endemicity and distributional patterns (Fattorini, 2002). The lack of endemic species of the *Polistes* genus in the Tyrrhenian islands may suggest the occurrence of gene flow sustained both by active and anthropic dispersion. Due to these immigrations, it can be assumed that similarities in epicuticular hydrocarbons observed among populations of the different sites are affected by current geographic factors. At least two lines of evidence have to be considered to explain the findings of this study: i) the relatively small distances between Capraia and Corsica and among Elba, Giglio, and the Tuscany coast (Figure 1), and ii) the main direction of windstorms (from West for Capraia and from South and North-East for Giglio and Elba) (Anonymous, 1978). These factors could explain a stronger genetic similarity within the population of the two above mentioned blocks that is reflected by the cuticular hydrocarbon composition (Chapman et al., 1995; Clément and Bagnères, 1998; Singer et al., 1998). In many species of insects, as well as in *Polistes*, the composition of the epicuticular mixture is probably affected not only by genetics, but also by environmental factors (Singer et al., 1998; Etges and Ahrens, 2001). In *Drosophila*, environmental features can affect cuticular chemical composition in two different ways. First, a progressive change in hydrocarbon blend across generations may occur in response to

different laboratory rearing conditions (Toolson and Kuper-Simbron, 1989), while, second, short-term ecophenotypic changes can be found in response to different temperatures or foods (Markow and Toolson, 1990; Etges and Ahrens, 2001). Moreover, Buckely et al. (2003) found a correlation between vegetation and cuticular composition in the grasshopper *Chorthippus parallelus*, emphasizing the importance of environmental influences. Liang and Silverman (2000) have also shown that the Argentine ant acquires prey-derived hydrocarbons on the cuticle and uses them in nestmate recognition. Several studies show differences in cuticular profiles among species and populations of insects and plants (Chapman et al., 1995; Maffei, 1996; Haverty et al., 2000; Etges and Ahrens, 2001). Capraia wasps prey upon insects and forage on plants more similar to those present on Corsica than on the Italian mainland (which shares several biotic characteristics with Elba and Giglio islands). It is possible that the cuticular hydrocarbon composition of wasps reflects habitat similarities within the two biogeographic groups.

It is noteworthy that within each biogeographic group, wasp profiles from the same area did not cluster together; suggesting influencing factors other than geographic location (Sledge et al., 2001; Dapporto et al., 2004a). Several compounds were important in discriminating cuticular profiles from Capraia-Corsica and Elba-Giglio-Venturina. These were 2 linear alkanes, 13 monomethyl alkanes, and 9 dimethyl alkanes. Many authors have reported methyl-branched alkanes as important discriminating substances among different species, populations, and colonies of social insects (Nelson, 1993; Haverty et al., 2000). Moreover, Dani et al. (2001) found that in *P. dominulus* alteration of the cuticular mixture by the augmentation of some alkenes and methyl-branched alkanes affected nestmate recognition, whereas the supplementation of linear alkanes did not. Recent studies have demonstrated that *P. dominulus* is able to recognize conspecifics coming from different localities (Dapporto et al., 2004b). The occurrence of strong chemical differences among the populations in the hydrocarbons involved in recognition is probably at the base of the discrimination among wasps from different sites.

In summary, characterization of the CHCs from wasps reflects the current biogeographic paradigm for the Tyrrhenian area. Moreover, because chemical profiles of paper wasps are affected both by genetic and environmental factors, these signatures provide useful data not only on the single species, but also on the "wasp habitat" as a whole, and could represent a valuable biogeographic tool.

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SUBMICRO SCALE NMR SAMPLE PREPARATION FOR VOLATILE CHEMICALS

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Abstract—A simple, inexpensive, and highly efficient NMR sample preparation technique for volatile chemicals has been devised using a micropreparative GC system. The recovery efficiency of a volatile chemical using this technique was >80% with sample sizes of 0.05 to 0.5 μg. The purity of the acquired NMR samples was sufficient for high sensitive NMR analyses including two dimensional experiments.

Key Words—Submicro sample, preparative GC, volatile semiochemicals, NMR.

INTRODUCTION

NMR has been a powerful tool for identification of semiochemicals in chemical ecology. Recently, the performance of NMR instruments has been remarkably improved, and it is now possible to conduct NMR analyses on microscale samples. However, there are several practical problems in preparing NMR samples with volatile semiochemicals, such as insect pheromones, that are available only in minute quantities (Webster and Kiemle, 1998).

Generally, volatile semiochemicals are obtained from various sources as extracts using organic solvents. These crude extracts are purified by means of various chromatographical techniques to isolate the NMR sample for structural elucidation. A major obstacle in preparing good NMR samples is removal of inevitable

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impurities: residual solvents, contaminants accompanying the purification processes, water, and other nontarget components. These impurities generate many spurious and sometimes significant signals in the NMR spectrum, particularly with submicro- to microgram scale samples. This "noise" makes interpretation of the spectra difficult and oftentimes impossible. The best way to avoid those problems is to prepare NMR samples using a preparative GC system (Webster and Kiemle, 1998). However, there are still some technical and practical problems: poor efficiency of sample recovery at the preparative GC step, the possibility of contamination in the NMR sample preparation step, and inefficient cost performance with the use of either an expensive and complex commercial instrument or a time-consuming, custom-built preparative GC system that would be used on rare occasions.

We report here a simple, inexpensive, and highly efficient NMR sample preparation technique for volatile chemicals in submicrogram quantities.

METHOD AND MATERIALS

Preparative Gas Chromatography System. An HP 5890 gas chromatograph modified as a GC-EAD system (Nojima et al., 2003) was converted to a preparative GC. The EAD port was used for the sample collection port. This port was unchanged, except that a 0.7 mm i.d. aluminum tube (Small Parts, Inc., Miami Lakes, FL, USA) was used as a liner to hold the megabore sample collection capillary tube in the port. A regular GC system equipped with a FID port could be used for this with minor modification of the port.

A schematic diagram of the GC configuration is shown in Figure 1. A nonpolar Equity-1 megabore capillary column (1.5-μm film thickness, 0.53 mm i.d. $\times$ 5 M,

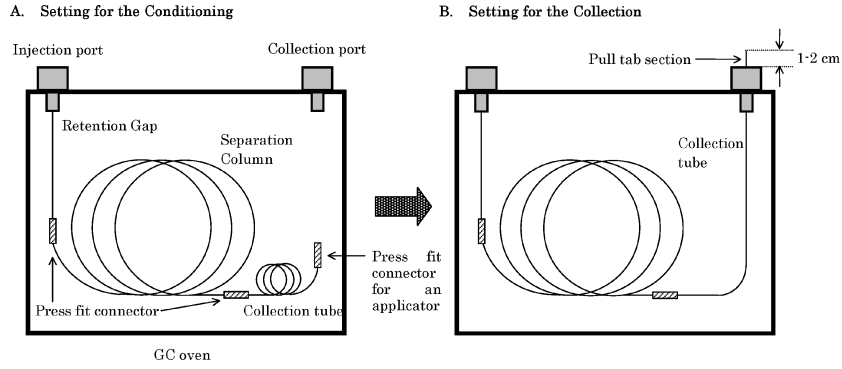

FIG. 1. Settings of micropreparative gas chromatography for conditioning and sample collection.

Supelco, Inc., Bellefonte, PA, USA) was used as a separation column, and two short inactive columns (0.53 mm i.d., no stationary phase, Alltech Associates, Inc., Deerfield, IL, USA) were connected to the column with press fit connectors (Alltech Associates, Inc., Deerfield, IL, USA). One of the inactive columns was the retention gap (2 m) at the front of the separation column, whereas the other was the sample collection tube (40 cm) at the end of the column (Figure 1A). The collection tube was prerinsed with the NMR solvent prior to conditioning. Additionally, another connector used as an applicator for NMR sample preparation was attached to the end of the collection tube to preclean it. After conditioning, the applicator was removed, and the end of collection tube was fixed to the collection port for sample collection (Figure 1B) (see point 1 in NMR Sample Preparation section, for details).

Sheath for Sample Collection. A detachable sheath was designed for efficient sample trapping into the collection tube (Figure 2). Telescope type aluminum tubings were obtained from Small Parts, Inc. (Miami Lakes, FL, USA). Other tubings, fittings, and parts were purchased from Supelco Inc. (Bellefonte, PN, USA) or Alltech Associates, Inc. (Deerfield, IL, USA). The sheath generates a gradual cooling zone along the collection tube, which is crucial to high recovery efficiency (Heath and Dueben, 1998). The tubes are heated from the bottom by the high temperature of the collection port, and they are cooled from the top by an ice bath.

GC Conditions. Nitrogen was used as the carrier gas at a head pressure of 1.0 psi and a flow rate of 8.0 ml/min. The time for splitless injection was 1.0 min. The oven temperature was set initially at 40°C for 2 min, increased at 10°C/min to 250°C, and held for 10 min. The injector and collection port temperatures were set at 150°C, and the septum purge flow rate was set at 1.5 ml/min with a total flow rate of 100 ml/min. The model compound eluted at ca. 155°C under these conditions. Since this technique has been developed for a thermally and chemically unstable insect sex attractant pheromone, the injection and collection port temperatures were set quite low. However, in a preliminary experiment, it was confirmed that significant loss of sample introduction into the column at the injection port did not occur under these GC conditions.

Chemicals. Diethyl phthalate (DEP, MW 220, b.p. 295°C) was used as a model compound. It has a similar molecular weight and retention time on a nonpolar column to a targeted insect pheromone; it also exhibits similar column chromatographical behavior. Standard solutions of DEP at 0.01–1.0 μg/μl in methylene chloride were prepared, and one to several microliters of sample volumes were injected in the GC. Benzene-*d*6 was purchased from Cambridge Isotope Laboratories, Inc., (Andover, MA, 99.96% deuteration) and used as an NMR solvent.

NMR Instrumentation. NMR spectra were measured with a Bruker AVANCE 600 MHz spectrometer. A 5-mm coaxial tube with an inner sample tube (1.5 mm

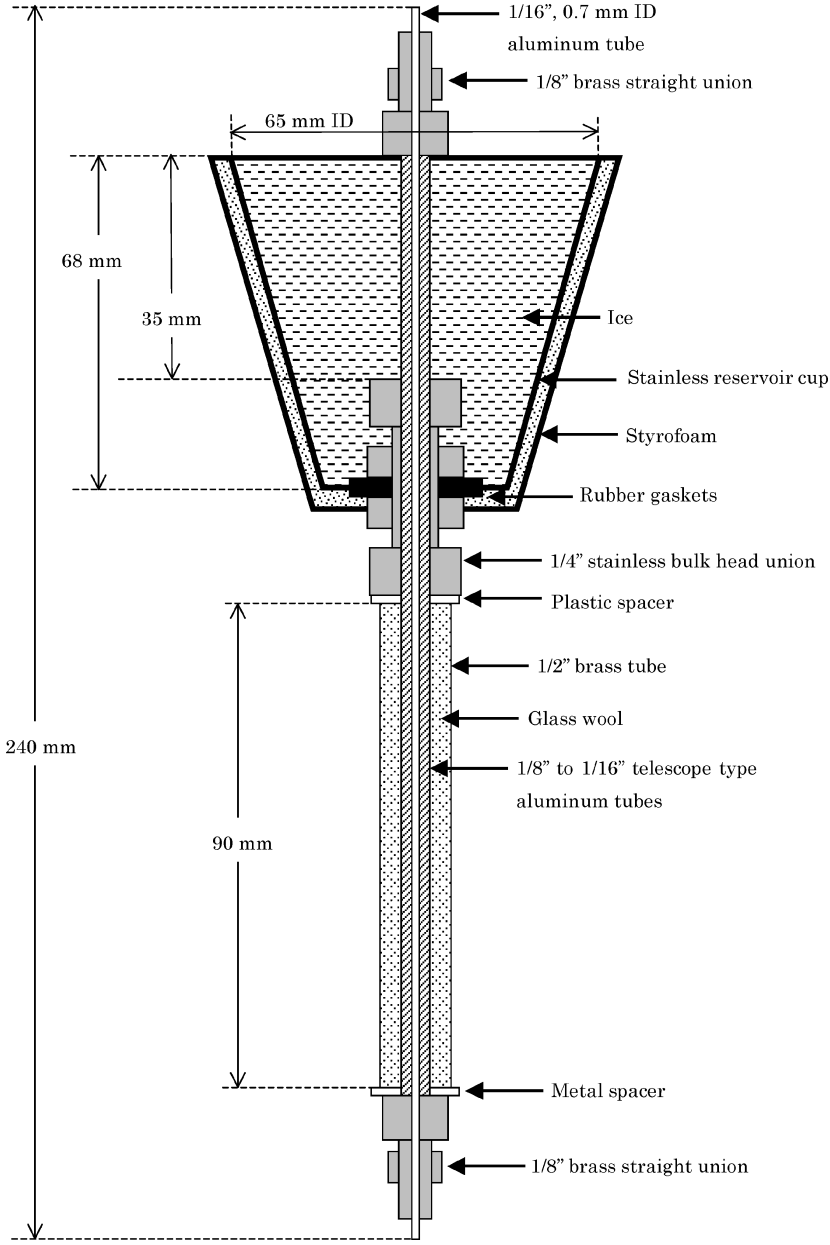

FIG. 2. Diagram of the sheath for sample collection with the micropreparative gas chromatography.

i.d., 2.52 mm o.d.) was used as a microcell NMR tube (Wilmad-Labglass, Co., Buena, NJ, USA). The signal of a trace amount of water, which is an unavoidable contaminant in NMR samples, was used as a reference (Silverstein and Webster, 1998).

NMR Sample Preparation

1. Conditioning of the System: Column, Tubes, and Connectors. A press fit connector rinsed with the NMR solvent was attached to the end of the collection tube (Figure 1A) for the conditioning phase. A GC program was run under the same conditions as for the preparative GC run. After conditioning, the connector was removed and kept in a clean vial until used for NMR sample preparation (see point 4).

The end of the collection tube was fixed to the collection port with a regular column nut just tight enough to hold the tube with its end protruding ca. 1-2 cm from the outlet (Figure 1B) to serve as a pull tab in the sample collection step (see point 3).

2. Preconditioning of the Sheath. The upper end of the aluminum tube (i.d. 0.7 mm, Figure 2) of the sheath was plugged with a GC septum. This prevents moisture and other contaminants from condensing inside the tube during preconditioning. The reservoir cup was filled with ice and the sheath attached to the EAD port of another GC-EAD system similar to the EAD port of the preparative GC. The collection port was heated to the same temperature as the collection port of the preparative GC. An alternative heating device could be used for this purpose. Preconditioning creates a thermal-gradation along the inside of the aluminum tubes of the sheath from the lower to upper sections. The sheath was kept in the preconditioning state until used.

3. Sample Collection. A schematic diagram of the sample collection is shown in Figure 3. A sample injection was made and the GC program started simultaneously (Figure 3A). Just prior to the retention time of the standard (DEP) or the semiochemical, the collection tube was gently and quickly drawn out of the collection port with the pull tab section until the press fit connector reached the column nut of the collection port (Figure 3B). Premarkings on the collection tube with a permanent marker assist in determining how far the collection tube should be drawn from the port. Immediately, the preconditioned sheath was set on the exposed collection tube and mounted on the collection port (Figure 3C). When the sample collection window was over, the sheath was removed (Figure 3D), and the collection tube pulled out forcefully to detach it from the press fit connector (Figure 3E). The collection tube was immediately set up for the rinsing step (point 4).

4. Preparation of NMR Sample. A simple diagram of the NMR sample preparation step is shown in Figure 4. Prior to rinsing the condensed sample

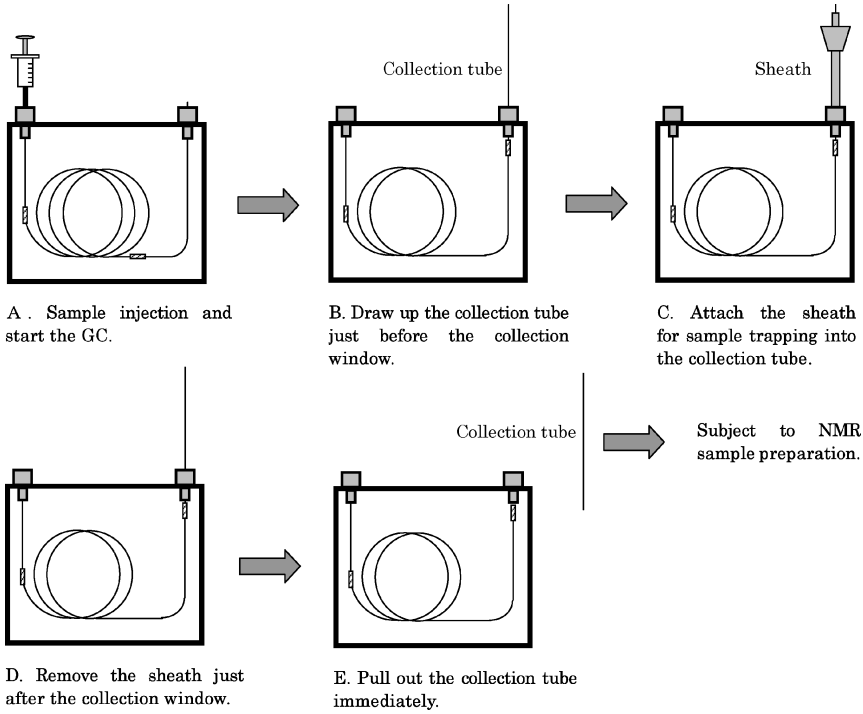

FIG. 3. A schematic protocol of the sample collection with the micropreparative gas chromatography.

from the collection tube, the pull tab section was carefully cut off with a column cutter to avoid contamination from this section. Various materials could accumulate in this exposed tab due to its low temperature, and the outside could be contaminated with fingerprints. In a preliminary experiment, the model compound condensed mainly in the section 10–30 cm from the outlet side of the collection tube. Thus, the loss of sample would be negligible by removing the tab.

The press fit connector, preconditioned as an applicator (see point 1), was attached to the cut side of the collection tube. The collection tube and an NMR tube were then set on a stand at an appropriate position for rinsing (Figure 4). The collection tube was rinsed with several portions of the NMR solvent *via* the applicator using a well cleaned and dried 10 μl GC syringe. The NMR tube was immediately sealed and subjected to NMR analyses. A few NMR control collections with solvent and blank injections were made at the same collection window time as the model compound.

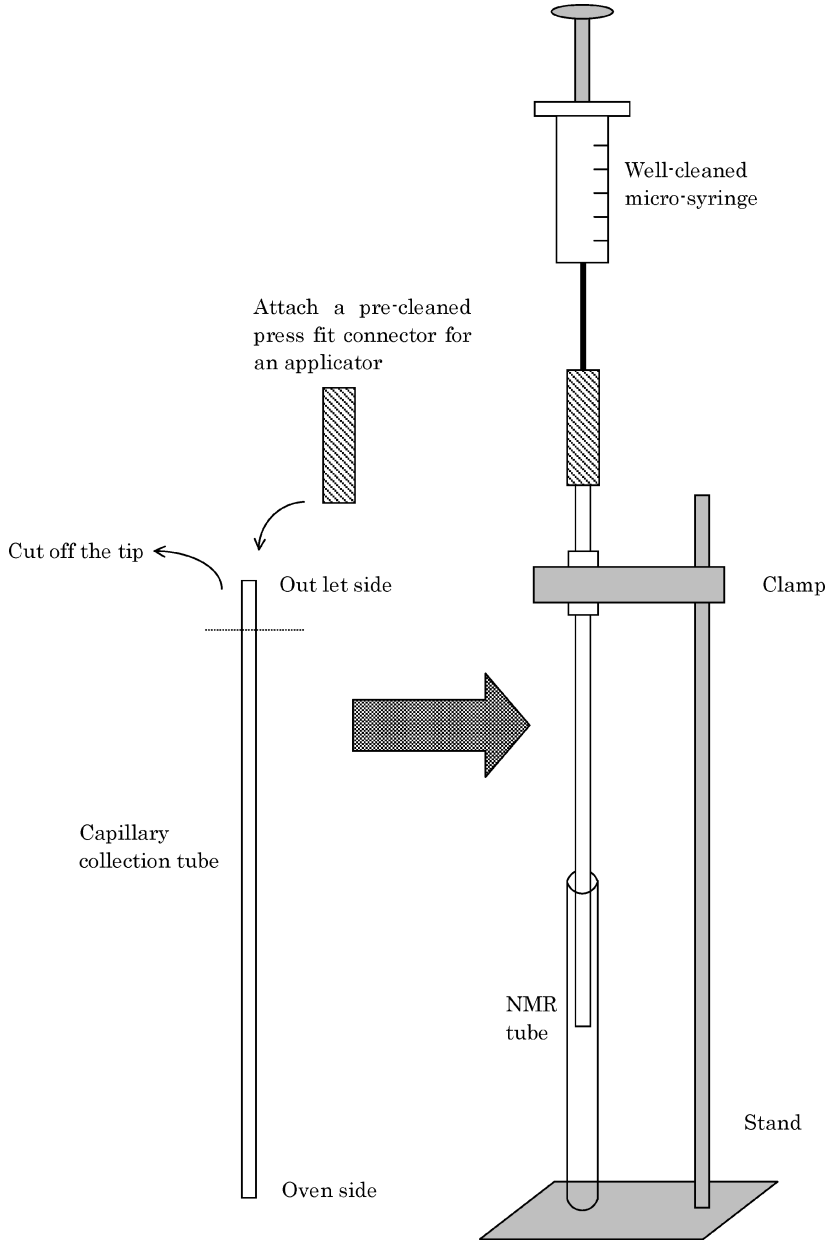

FIG. 4. A schematic diagram of the NMR sample preparation steps with the collection tube.

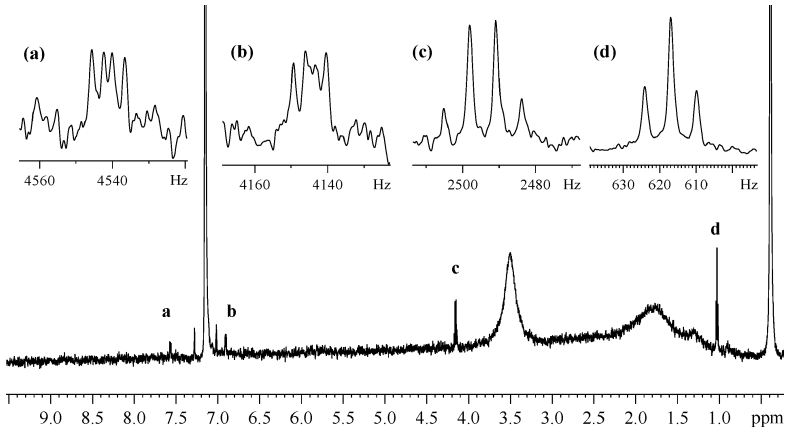

FIG. 5. ^{1}H-NMR spectrum of diethyl phthalate at ca. 0.5 μg in deuterium benzene, prepared by the micropreparative gas chromatography technique.

RESULTS AND DISCUSSION

Recovery efficiency of the model compound (DEP) using the micropreparative GC technique was >80% with sample injection sizes ranging from 0.05 from 0.5 μg (mean recovery efficiency: 97%, $N = 6$).

A representative ^{1}H-NMR spectrum of 0.5 μg of DEP at ca. 10 ng/μl in deuterium benzene is shown in Figure 5. The signals of DEP were observed clearly at 7.58(m), 6.91(m), 4.16 (q), and 1.03(t) ppm. A strong signal from the solvent (benzene-*d*6) at 7.15 ppm and of water appeared at 0.39 ppm, whereas no significant signal from impurities was found. Although a few minor broad signals at 3.50 and 1.77 ppm were observed, these seem to originate in the NMR solvent itself and can be negligible. The purity of the samples was sufficient for high sensitive NMR analyses including two dimensional COSY experiments with ^{1}H-^{1}H correlations clearly delineated (spectra not shown).

This microcollection technique has been devised for single sample collection in a GC run and is preferable for a partially purified sample. However, it is feasible for multiple sample collections within a run making few modifications to the collection protocol.

This technique has been used for the collection of a few micrograms of a thermally and chemically unstable insect sex pheromone with great success. The purity of the resultant NMR sample was sufficient for conducting two dimensional NMR experiments. The result will be published elsewhere. Although the GC conditions in this study were designed for a specific unstable volatile compound,

this technique could be used with other GC conditions for most volatile semiochemicals.

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PHYTOTOXIN PRODUCTION AND PHYTOALEXIN ELICITATION BY THE PHYTOPATHOGENIC FUNGUS *Sclerotinia sclerotiorum*

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Abstract—The fungus *Sclerotinia sclerotiorum* (Lib.) de Bary causes rot disease in a vast range of plant families, including Cruciferae (Brassicaceae). We investigated the production of phytotoxins by *S. sclerotiorum* by using a bioassay-guided isolation, as well as the phytoalexins produced by the resistant wild crucifer *Erucastrum gallicum* under elicitation by *S. sclerotiorum* and other agents. We established for the first time that *S. sclerotiorum* produces a somewhat selective phytotoxin, sclerin, which is phytotoxic to three cruciferous species (*Brassica napus*, *B. juncea*, and *Sinapis alba)* susceptible to Sclerotinia stem rot disease, causing severe necrosis and chlorosis, but not to a resistant species (*Erucastrum gallicum*). In addition, we have shown that oleic acid, the major fatty acid isolated from sclerotia of *S. sclerotiorum* is responsible for the toxic activity of extracts of sclerotia to brine shrimp larvae (*Artemia salina*). Phytoalexin elicitation in leaves of *E. gallicum* led to the isolation of three known phytoalexins: indole-3-acetonitrile, arvelexin, and 1-methoxyspirobrassinin. Considering that resistance of *E. gallicum* to *S. sclerotiorum* is potentially transferable to *B. rapa*, a susceptible canola species, and that arvelexin, and 1-methoxyspirobrassinin are not produced by *B. rapa*, these phytoalexins may become useful markers for resistance against *S. sclerotiorum*.

Key Words—Antifungal, *Brassica napus*, *B. juncea*, *B. rapa*, Cruciferae, *Erucastrum gallicum*, phytoalexin, phytotoxin, plant pathogen, sclerin, *Sclerotinia sclerotiorum*, stem rot.

INTRODUCTION

The fungus *Sclerotinia sclerotiorum* (Lib.) de Bary causes rot disease in a vast range of plant families, including Brassicaceae or Cruciferae, and is considered an important problem worldwide (Kohli et al., 1995; Bom and Boland, 2000). The

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fungus produces sclerotia, overwintering structures that can remain in the soil for many years. The dark pigmentation of sclerotia is due to melanin, a substance believed to play an important role in protecting fungi from biological degradation and environmental conditions (Starratt et al., 2002). Yield losses due to Sclerotinia stem rot in the oilseeds canola and rapeseed (*Brassica napus, B, rapa*) can cause losses up to 50% depending on environmental and weather conditions (Lefol et al., 1997). The lack of host specificity makes breeding of resistant varieties difficult (Saharan, 1993). Importantly, however, a new source of genetic resistance to *S. sclerotiorum* was discovered in *Erucastrum gallicum,* a wild crucifer potentially useful in oilseed breeding programs (Lefol et al., 1997). In addition, due to increasing problems and concerns over the use of fungicides, there has been strong interest in the biocontrol of *Sclerotinia* diseases. Nevertheless, progress in the development of biocontrol products has been slow, due to insufficient knowledge in the areas of inoculum production and their efficacy under various environmental conditions. Consequently, integration of effective control methods, such as conventional and biological methods, appears to be the best strategy for managing this important disease (Bardin and Huang, 2001).

Our approach to design strategies for controlling plant fungal diseases requires a chemical-ecological understanding of the plant and the pathogen cultured in isolation and in direct contact. Necrotrophic phytopathogens such as *S. sclerotiorum* are expected to produce phytotoxins to damage plant tissues and facilitate colonization, while in response to this attack plants synthesize phytoalexins. Phytoalexins are secondary metabolites produced *de novo* by plants in response to diverse forms of stress including microbial infection, UV irradiation, and salts. Both fungi and plants can counterattack by producing enzymes capable of detoxifying either phytotoxins, in the case of plants, or phytoalexins in the case of pathogens. While phytoalexins are an important part of the plants' defense mechanism (Smith, 1996, Brooks and Watson, 1985) phytotoxins are virulence/pathogenicity factors of phytopathogenic fungi (Graniti, 1991). Recent work has shown that *S. sclerotiorum* is able to detoxify some important crucifer phytoalexins and their precursors (Pedras and Ahiahonu, 2002; Pedras and Montaut, 2003), and produce oxalic acid as a pathogenicity factor (Cessna et al., 2000). To the best of our knowledge no phytotoxins from *S. sclerotiorum* have been reported. Nonetheless, *S. sclerotiorum* was shown to produce a variety of polyketides (Morita and Aoki, 1974) such as sclerin (**1**), (Satomura and Sato, 1963), sclerolide (**2**), sclerotinin A (**3**) (Kubota et al., 1966), sclerotinin B (**4**) (Sassa et al., 1968), sclerone (**5**) (Suzuki et al., 1968), and isosclerone (**6**) (Morita and Aoki, 1974) (Figure 1). Some of these metabolites have interesting plant hormone type activity; for example, **3** and **4** promote the growth of rice seedlings at low concentration (Tokoroyama et al., 1968), whereas **6** stimulates the root elongation of rice seedlings at concentrations of 1–10 ppm, and inhibits the growth of shoots and roots at higher concentrations (>50 ppm). Sclerin (**1**) (Marukawa et al., 1975) plays a role in induction of sclerotium formation and melanogenesis in *Sclerotinia* species.

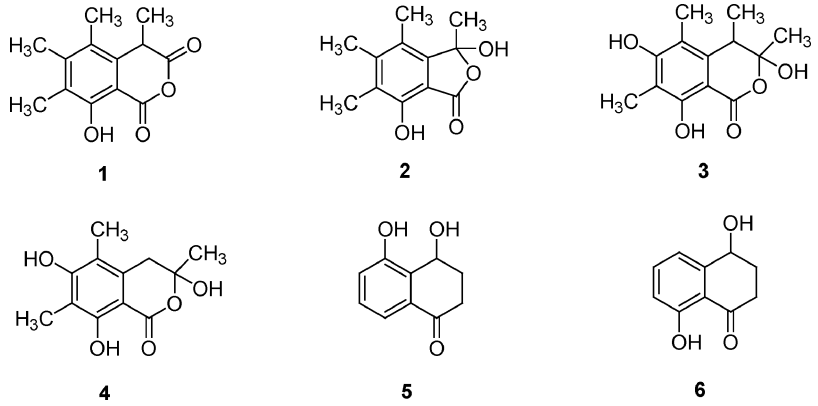

FIG. 1. Secondary metabolites isolated from liquid cultures of *Sclerotinia sclerotiorum:* sclerin (**1**) (Satomura and Sato, (1963); Marukawa et al., 1975), sclerolide (**2**), sclerotinin A (**3**) (Kubota et al., 1966), sclerotinin B (**4**) (Sassa et al., 1968), sclerone (**5**) (Suzuki et al., 1968), isosclerone (**6**) (Morita and Aoki, 1974).

We have investigated the production of phytotoxins by *S. sclerotiorum* using a bioassay-guided isolation, as well as the phytoalexins produced by the resistant wild crucifer *E. gallicum* under elicitation by *S. sclerotiorum* and other agents, and report here results of these studies.

METHODS AND MATERIALS

General Experimental Procedures. All chemicals and media were purchased from Sigma-Aldrich Canada Ltd., Oakville, ON. All solvents were HPLC grade and used as such, except for CH_2Cl_2 and $CHCl_3$that were redistilled. Remaining conditions were as previously reported (Pedras et al., 2002).

Analytical Methods. Analytical thin layer chromatography (TLC) was carried out on precoated silica gel TLC aluminum sheets (EM science, Kieselgel 60 F_{254}, 5 × 2 cm × 0.2 mm). Compounds were visualized under UV light (254/366 nm) and by dipping the plates in a 5% aqueous (w/v) phosphomolybdic acid solution containing 1% (w/v) ceric sulfate and 4% (v/v) H_2SO_4, followed by heating. Preparative thin layer chromatography (prep TLC) was performed on silica gel plates (EM science, 60 F_{254} or reversed-phase RP-18, 20 × 20 cm, 0.25 or 0.5 mm thickness). Compounds were visualized under UV light. Flash column chromatography (FCC) was performed on silica gel, Merck grade 60, mesh size 230–400, 60 Å or J. T. Baker C-18 reversed-phase silica gel, 40 μm. HPLC analysis was carried out with an high performance liquid chromatograph equipped with quaternary pump, automatic injector, and diode array detector (wavelength

range 190–600 nm), degasser, and a Hypersil ODS column (5 μm particle size silica, 4.6 i. d. $\times$ 200 mm), equipped with an in-line filter. Mobile phase: 75% H_2O–25% CH_3CN to 100% CH_3CN, for 35 min, linear gradient, and a flow rate 1.0 ml/min.

Specific rotations, $[\alpha]_D$ were determined at ambient temperature on a Perkin-Elmer 141 polarimeter using a 1 ml, 10^{-2} cm path length cell; the units are 10^{-1} deg cm^2 g^{-1}, and the concentrations (c) are reported in gram per 100 ml. NMR spectra were recorded on a Bruker AMX 300 or Avance 500 spectrometers. For ^{1}H NMR (300 or 500 MHz), the chemical shifts (δ) are reported in parts per million (ppm) relative to TMS. The δ values were referenced to $CDCl_3$ ($CHCl_3$ at 7.27 ppm), CD_2Cl_2 ($CHDCl_2$ at 5.32 ppm), CD_3CN (CD_2HCN at 1.94 ppm), CD_3SOCD_3 (CHD_2SOCD_3 at 2.50 ppm), or CD_3OD (CHD_2OD at 3.31 ppm). First-order behavior was assumed in analysis of ^{1}H NMR spectra, and multiplicities are as indicated by one or more of the following s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad. Spin coupling constants (J values) are reported to the nearest 0.5 Hz. For ^{13}C NMR (75.5 or 125.8 MHz), the chemical shifts (δ values) were referenced to $CDCl_3$ (77.23 ppm), CD_2Cl_2 (54.00 ppm), CD_3CN (118.69 ppm), CD_3SOCD_3 (39.51 ppm), or CD_3OD (49.15 ppm). The multiplicities of ^{13}C signals refer to the number of attached protons: s = C, d = CH, t = CH_2, q = CH_3, and were determined based on HMQC correlations and magnitude of J values. Mass spectrometry (MS) (high resolution (HR), electron impact (EI)) data were obtained on a VG 70 SE mass spectrometer using a solids probe. Gas chromatography—mass spectrometry (GC-MS) data were obtained on a Fisons GC 8000 series model 8060 connected to the VG 70 SE mass spectrometer. Fourier transform infrared (FTIR) spectra were recorded on a Bio-Rad FTS-40 spectrometer. Spectra were measured by the diffuse reflectance method on samples dispersed in KBr. Ultraviolet (UV) spectra were recorded on a Varian Cary 100 spectrophotometer using a 1-cm quartz cell.

Plant Material and Growth. Seeds of canola (*Brassica napus*) cv. Westar, white mustard (*Sinapis alba*) cv. Ochre, brown mustard (*B. juncea*) cv. Cutlass, and dog mustard (*E. gallicum*, a wild crucifer) were obtained from Plant Gene Resources, Agriculture and Agric-Food Canada Research Station, Saskatoon, SK. The seeds were sown in commercial potting soil mixture, and plants were grown in a growth chamber, under controlled environmental conditions (20/18°C with 16/8 L/D cycle) for 2–5 wk.

Fungal Isolates, Cultures, and Extractions. Fungal isolates were obtained from Agriculture and Agric-Food Canada Research Station, Saskatoon, SK. Cultures of *Sclerotinia sclerotiorum* clones # 33 and # 67 and *Rhizoctonia solani* AG-2 were maintained on potato dextrose agar (PDA) cultures. Liquid cultures of *S. sclerotiorum* were initiated with five sclerotia per 100 ml of potato dextrose broth (PDB) media in 250 ml Erlenmeyer flasks. After incubation on a shaker

(110 rpm) at 20°C for 14 d, the mycelial mat was filtered off, and the broth was extracted with EtOAc. The combined EtOAc extracts were dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure. Liquid cultures were also prepared in minimal media (14 d incubation) (Pedras et al., 1997) and Czapek-Dox media (50 d incubation) and treated the same way as described above. Sclerotia (275 g) of *S. sclerotiorum* clone # 33 collected from 3–4 wk-old agar plates, were mixed with water (500 ml) and ground (using a commercial blender). The fine paste was extracted first with EtOAc followed by butanol. The two extracts were dried over anhydrous Na_2SO_4, and the solvents removed under reduced pressure.

Isolation of Phytotoxic Metabolites from Sclerotinia sclerotiorum. To establish the optimum incubation time for the production of phytotoxic metabolites, ethyl acetate and butanol broth extracts of 7-, 14-, and 21-day-old cultures of *S. sclerotiorum* were assayed by using a leaf puncture bioassay and analyzed by HPLC. Two-wk-old plants (*B. napus*, *B. juncea*, and *S. alba*) susceptible to, and 5-wk-old plants (*E. gallicum*) resistant to Sclerotinia stem rot disease were used for bioassays. The EtOAc extract obtained from 17 l of liquid cultures (PDB) of *S. sclerotiorum* (1.02 g) was dissolved in CH_2Cl_2–MeOH (95:5, 1 ml) and fractionated by FCC on silica gel with gradient elution: CH_2Cl_2–MeOH (95:5, 8 fractions of 100 ml), CH_2Cl_2–MeOH (90:10, 1 fraction of 100 ml), CH_2Cl_2–MeOH (80:20, 1 fraction of 100 ml), CH_2Cl_2–MeOH (50:50, 1 fraction of 100 ml), and MeOH (1 fraction of 100 ml). All 12 fractions were analyzed by HPLC and TLC and bioassayed on *B. napus*, *B. juncea*, and *S. alba*. Phytotoxic fractions 5–8 (HPLC prominent peak at $R_t = 24$ min) were combined (138 mg) and fractionated by RP-FCC (C-18 silica gel with gradient elution: acetonitrile–water, 1:1, 15 fractions of 20 ml; acetonitrile, 100 ml, and methanol, 100 ml). Fractions 10–12 (HPLC peak at $R_t = 24$ min) showed phytotoxicity and were combined. Further separation by preparative TLC (silica gel, CH_2Cl_2–MeOH, 95:5) yielded a single compound showing phytotoxicity (sclerin **1**, 50 mg).

*Spectroscopic Data for Sclerin (**1**).* HPLC $R_t = 24.0$ min; ^{1}H NMR (500 MHz, $CDCl_3$): δ 10.78 (s, 1H, D_2O exchangeable), 4.18 (q, $J = 7.5$ Hz, 1H), 2.32 (s, 3H), 2.26 (s, 3H), 2.20 (s, 3H) 1.59 (d, $J = 7.5$ Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 169.2 (s), 166.7 (s), 159.2 (s), 148.2 (s), 134.9 (s), 125.0 (s), 124.4 (s), 101.8 (s), 39.1 (d), 22.7 (t), 17.9 (t), 14.9 (t), 12.3 (t); HRMS-EI *m/z*: measured 234.0890 (M^+, calcd. 234.0892 for $C_{13}H_{14}O_4$); MS-EI *m/z* (relative intensity): 234 (M^+, 54), 206 (100), 191 (13), 163 (20), 147 (12), 91 (12); FTIR ν_{max}: 3203, 2987, 2933, 2872, 1795, 1694, 1606, 1455, 1331, 1267, 1136, 1103, 1011, 946, 796, 730, 560 cm^{-1}; UV (EtOH) λ_{max} (log ε): 212 (4.3), 264 (3.8), 328 (3.5).

Toxic Compounds from Sclerotia of S. sclerotiorum. The EtOAc extract (390 mg) of sclerotia of *S. sclerotiorum* was subjected to FCC on silica gel with

gradient elution: CH_2Cl_2–MeOH (99:1, 10 fractions of 100 ml), CH_2Cl_2–MeOH (95:5, 1 fraction of 100 ml), CH_2Cl_2–MeOH (90:10, 1 fraction of 100 ml), CH_2Cl_2–MeOH (80:20, 1 fraction of 100 ml), CH_2Cl_2–MeOH (50:50, 1 fraction of 100 ml), and MeOH wash (1 fraction of 100 ml). In all, 15 fractions were collected. The fractions were bioassayed on the brine shrimp larva, *Artemia salina*. Fractions 3 and 4, showed toxicity and were combined (40 mg) and further subjected to preparative TLC using CH_2Cl_2–MeOH (99:1, developed three times) to give a fraction (12.2 mg) that turned out to be a mixture of fatty acids. This fraction (2 mg) was treated with excess ethereal diazomethane at room temperature for 3 hr. The solvent was then removed under reduced pressure to give the methyl esters of fatty acids (2.6 mg). This was subjected to GC-MS analysis, and the amount of each fatty acid present was estimated. Oleic, stearic, and palmitic acids (2 mg each; purchased from Sigma-Aldrich Co.) were separately treated with excess ethereal diazomethane at room temperature for 3 hr and subsequently subjected to GC-MS analysis. GC-HREIMS was used to identify the various fatty acid methyl esters.

Leaf Puncture Assay. To ensure that penetration of the test solution in the leaf tissue at the wound site was uniform for the four species (*B. napus* leaves are thicker, due to a protective layer of wax covering the surface), the leaf surface was scratched or punctured, and the test solutions were applied to these sites (Vurro et al., 1998). The phytotoxic effects of extracts and sclerin (**1**) were evaluated by measuring the size of lesions on leaves. The toxicity of the extracts and metabolites was tested at different concentrations (Table 1). Plants were observed daily up to 7 d after droplet application to evaluate leaf damage or other changes.

Fully expanded leaves of the plants described above were scratched and/or punctured (four punctures per leaf, and two leaves per plant). Droplets (10 μl) of each test solution (composed of 50% aqueous methanol) were applied at each puncture. Six concentrations for sclerin (**1**) (1×10^{-3} to 1×10^{-5} M) and five concentrations for oxalic acid (5×10^{-2} to 2×10^{-4} M) as shown in Table 1, were prepared by serial dilution, broth extract of liquid culture of *S. sclerotiorum* (1 mg/ml and 2 mg/ml), chromatographic fractions (each 1 mg/ml) and sclerotia extract (1 mg/ml and 2 mg/ml) were applied. Symptom appearance was observed daily up to 7 d after droplet application. The diameter of the leaves was measured with a ruler. Each treatment was repeated at least three times. Solutions of 50% aqueous methanol were applied as control (Pedras et al., 2000a). Statistical analyses were conducted using a one-tailed, paired-sample *t*-test.

Leaf Uptake Assay. Four toxin concentrations of sclerin (**1**), oxalic acid (1×10^{-3}, 5×10^{-4}, 1×10^{-4}, and 1×10^{-5} M), and broth extract (0.6 mg/1 ml) in 2% acetonitrile were prepared by serial dilution. Leaves were cut at the base of their petiole, and each leaf was immediately placed in a 1.5 ml Eppendorf tube containing assay solution (1 ml per tube per leaf). After the test solution was

TABLE 1. PHYTOTOXIC ACTIVITY[a] OF SCLERIN (**1**), OXALIC ACID, BROTH, AND SCLEROTIA EXTRACTS OF *Sclerotinia sclerotiorum*

Phytotoxin	Concentration	*B. napus*[b,c,d] cv. Westar	*B. juncea*[b,c,d] cv. Cutlass	*S. alba*[b,c,d] cv. Ochre	*E. gallicum*[b,d,e]
Sclerin (**1**)	1×10^{-3} M	$3.9 \pm 0.2^{***}$	$4.2 \pm 0.2^{***}$	$4.0 \pm 0.2^{***}$	—*
	5×10^{-4} M	$2.7 \pm 0.1^{**}$	$2.8 \pm 0.2^{**}$	$2.8 \pm 0.2^{**}$	—*
	3×10^{-4} M	$1.7 \pm 0.1^{**}$	$1.8 \pm 0.2^{**}$	$1.8 \pm 0.1^{**}$	—*
	2×10^{-4} M	—*	—*	—*	—*
Oxalic acid	1×10^{-3} M	—*	—*	—*	—*
	5×10^{-4} M	—*	—*	—*	—*
	3×10^{-4} M	—*	—*	—*	—*
	2×10^{-4} M	—*	—*	—*	—*
Broth extract (14-day-old)	2.0 mg/ml	$4.4 \pm 0.2^{***}$	$4.5 \pm 0.3^{***}$	$4.5 \pm 0.2^{***}$	—*
	1.0 mg/ml	$2.8 \pm 0.2^{**}$	$3.0 \pm 0.1^{**}$	$2.9 \pm 0.2^{**}$	—*
Broth extract (21-day-old)	2.0 mg/ml	$3.5 \pm 0.2^{***}$	$3.6 \pm 0.1^{***}$	$3.5 \pm 0.2^{***}$	—*
	1.0 mg/ml	$2.2 \pm 0.1^{**}$	$2.3 \pm 0.2^{**}$	$2.2 \pm 0.1^{**}$	—*
Sclerotia extract	2.0 mg/ml	—*	—*	—*	—*
	1.0 mg/ml	—*	—*	—*	—*

[a] *Brassica napus* cv. Westar (susceptible), *B. juncea*, cv. Cutlass (susceptible) *Sinapis alba* cv. Ochre (susceptible) and *Erucastrum gallicum*, wild (resistant) after 7 d of incubation.

[b] Leaves scratched or punctured with needle and compound (in 50% aqueous methanol) applied with micropipette (10 μl); leaves scored after 1 wk; scores represent diameter of lesions;—: no lesion as in control experiment. Results are the mean of three independent experiments (±SD) with a minimum of ten replicates. Leaves scratched or punctured showed similar lesions.

[c] Two-week-old plants.

[d] P values denote the significant difference of the mean of diameter of lesions (mm) for test solutions containing extract or compound over controls. $^{*}P > 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Values were obtained using a one-tailed, paired-sample, t-test.

[e] Five-week-old plants.

taken up, an aqueous solution of 6-benzylaminopurine (1×10^{-5} M) was added to each tube (to delay leaf chlorosis), and leaves were incubated under constant light. Three independent experiments were carried out, each one in duplicate. Symptoms were observed daily up to 7 d (Pedras et al., 2000a).

Spray Assay. Two–five Week old plants *(B. napus, B. juncea*, *S. alba*, and *E. gallicum*) grown as described above were uniformly sprayed, using a hand sprayer, with 15 ml of 50% aqueous methanol solution of broth extract (3 mg/25 ml) or 15 ml of 50% aqueous methanolic solution of sclerin (**1**, 5×10^{-4} M), oxalic acid (5×10^{-4} M), or 15 ml of 50% aqueous methanol as control (Vurro et al., 1998). The pots were kept in the growth chamber at 24°C for 7 d. Plants were examined visually for chlorosis and necrosis.

Brine Shrimp Lethality Assay. Eggs of brine shrimp (*Artemia salina*) were incubated in a Petri dish containing 0.33 g of NaCl in 100 ml of water in the presence of fluorescent light (24 ± 2°C for 48 h). The EtOAc extract of sclerotia of *S. sclerotiorum* clone # 33 (10 mg), the fraction composed of fatty acids (10 mg), and the EtOAc extract of liquid cultures of *S. sclerotiorum* clone # 33 (10 mg) were assayed at three concentrations (1 mg/ml, 0.1 mg/ml, and 0.01 mg/ml). Three toxin concentrations of sclerin (**1**), oleic acid, palmitic acid, and stearic acid (5×10^{-4}, 10^{-4}, 10^{-5} M) were prepared in serial dilution in DMSO (10 μl) saline aqueous solution (total volume 1 ml). Brine shrimp larvae (about 10–15 in 1 ml saline solution) were transferred into each vial, and the vials were capped and kept at 24°C (fluorescent light). After 24 hr, the live brine shrimp larvae in each vial were counted. Three independent experiments were performed, each one in duplicate. Statistical analyses were conducted using a one-tailed, paired-sample *t*-test.

Elicitation of Phytoalexins with S. sclerotiorum. Leaves from 5-wk-old plants (*E. gallicum)* were excised with a sharp blade, the petioles were wrapped with premoistened cotton wool, and leaves placed in Petri plates (two leaves per plate). Each leaf was inoculated with five mycelium plugs placed upside down (4-mm cut from 3-d-old PDA plates of *S. sclerotiorum* clone # 33) and distributed evenly over the leaf surface. The Petri plates were sealed and incubated under constant fluorescent light for 7 d. After every 24 hr, leaves were frozen in liquid nitrogen, crushed with a glass rod, and extracted with EtOAc by shaking at 120 rpm for 30 min. The EtOAc was filtered, dried over anhydrous Na_2SO_4, and the solvent removed under reduced pressure. The extract was dissolved in 0.2 ml acetonitrile, filtered through a tight cotton plug, and analyzed by HPLC. Two independent experiments were carried out. The HPLC analysis of the EtOAc extracts indicated the presence of three compounds with $R_t = 11.2$, 13.1, and 16.9 not present in control samples. Quantification of the compounds was done using HPLC built standard calibration curves.

Elicitation of Phytoalexins with $CuCl_2$ or Oxalic Acid. Five-week-old plants (*E. gallicum*) were sprayed to the point of run-off with $CuCl_2$ or oxalic acid (2×10^{-3} M) solutions at 24-hr intervals for three d. Leaves were excised at 24 hr

intervals for 7 d; control leaves were harvested from separate plants at the same time and treated in a similar manner throughout. After various incubation periods, leaves were worked up as described above, and extracts were analyzed by HPLC. Three independent experiments were carried out.

*Elicitation of Phytoalexins with Sclerin (**1**) or Oxalic Acid.* Fully expanded leaves of 5-wk-old plants (*E. gallicum)* were punctured (four punctures per leaf, two leaves per plant), and droplets (10 μl) of sclerin (**1**) or oxalic acid (5×10^{-4} M) solutions in 50% aqueous methanol were applied at each punctured spot. Droplets of 50% aqueous methanol were applied as control. Leaves were excised at 24 hr intervals, were worked up as described above, and extracts were analyzed by HPLC. Control leaves were harvested from separate plants at the same time and treated in a similar manner throughout. Three independent experiments were carried out.

Antifungal Bioassays. The antifungal activity of arvelexin (**8**) and 1-methoxyspirobrassinin (**9**) was determined using the following mycelia radial growth bioassay. Solutions of each compound in DMSO (5×10^{-2} M) were used to prepare assay solutions in minimal media (5×10^{-4} M, 2.5×10^{-4} M, 5×10^{-5} M) in serial dilution; control solutions contained 1% DMSO in minimal media. Sterile tissue culture plates (12-well, 24-mm diam) containing test solutions and control solution (1 ml per well) were inoculated with mycelia plugs placed upside down on the centre of each plate (5-mm cut from 3-d-old and 7-d-old PDA plates of *S. sclerotiorum* clone # 33 and *Rhizoctonia solani* AG 2-1, respectively), and incubated under constant light for 7 d. The radial growth of mycelia was measured with a ruler daily for 1 wk. Three independent experiments were carried out each one in triplicate. Statistical analyses were conducted using a one-tailed, paired-sample *t*-test.

*Isolation of 1-Methoxyspirobrassinin (**9**).* Plants (40, 5-wk-old) were sprayed with $CuCl_2$ (2×10^{-3} M) solution to the point of run-off, three times at 24-hr intervals and allowed to stand for three d. Elicited leaves (180 g, fresh weight), were frozen in liquid nitrogen and crushed with a glxass rod, and extracted with EtOAc in a manner similar to that followed for the time course experiment. The EtOAc extract (1.2 g) was subjected to FCC (gradient elution, CH_2Cl_2, 100% to CH_2Cl_2-MeOH, 20:80). The fractions containing the HPLC peak at $R_t = 16.9$ min were combined (284 mg) and further fractionated by reverse phase FCC (gradient elution, CH_3CN-H_2O, 20:80) to CH_3CN, 100%) followed by reverse phase micro flash (2-cm plug of reverse phase C-18 silica gel in a Pasteur pipette, CH_3CN-H_2O (60:40).

*Spectroscopic Data of 1-Methoxyspirobrassinin (**9**).* HPLC $R_t = 16.9$ min.; $[\alpha]_D = 40.8$ (c 0.09, MeOH); ^{1}H NMR (500 MHz, CD_3CN): δ 7.42 (m, 2H), 7.17 (dd, $J = 7.5$, 7.5 Hz, 1H), 7.06 (d, $J = 7.5$ Hz, 1H), 4.60 (d, $J = 15.5$ Hz, 1H), 4.46 (d, $J = 15.5$ Hz, 1H), 3.99 (s, 3H), 2.62 (s, 3H);^{13}C NMR (125.8 MHz, CD_3CN): δ 171.0 (s, C-2), 163.2 (s, C-2′), 139.9 (s, C-7a), 130.3 (d, C-6), 126.9

(s, C-3a), 124.7 (d, C-4), 124.3 (d, C-5), 108.1 (d, C-7), 74.6 (t, C-4′), 72.6 (s, C-3), 63.9 (q,-OCH_3), 15.3 (q, -SCH_3); HRMS-EI m/z: measured 280.0336 (M^+, calcd. 280.0340 for $C_{12}H_{12}N_2S_2O_2$); MS-EI m/z (relative intensity): 280 (100, M^+), 252 (9, M^+ - CO), 249 (13, M^+ - C_2H_3NS), 234 (15, M^+ - SCH_3), 221 (25), 176 (52, M^+- [OCH_3 + C_2H_3NS]), 148 (50), 87 (37); FTIR ν_{max}: 2924, 2852, 1738, 1616, 1585, 1465, 1087, 944, 749 cm^{-1}; UV (CH_3CN) λ_{max} (log ε): 262 (3.7), 217 (4.4).

RESULTS AND DISCUSSION

Phytotoxins from Cultures of S. sclerotiorum. Inspection of the HPLC chromatograms of culture extracts indicated that clones # 33 and # 67 of *S. sclerotiorum* produced similar metabolites in each culture medium. The extracts of 14-d-old liquid cultures caused the most damage to the plants; large brown lesions in circular patterns (ca. 4.5-mm diam for extracts of 14-d-old culture broth *vs.* ca. 3.5-mm diam for 21-d-old culture broth) were observed on the three plant species tested (Table 1). These results indicated that the effects of phytotoxic metabolites peaked in 14-d-old liquid cultures. Furthermore, the HPLC chromatograms of ethyl acetate extracts of the cultures showed a peak with $R_t = 24.0$ min showing the highest intensity in extracts of 14-d-old cultures; this peak was not detected in the butanol extract.

In scale-up experiments, extracts (1.02 g) of 14-d-old PDB cultures (17 l) of *S. sclerotiorum* were obtained as described in the Methods and Materials section. The extracts were phytotoxic to *B. napus*, *B. juncea*, and *S. alba* (at 1 mg/ml); however, these extracts caused no damage to the leaves of *E. gallicum* at similar concentration (Table 1). The extract was subjected to FCC, and the bioactive fractions were combined and further purified. HPLC analyses coupled with bioassays enabled the isolation of a single phytotoxic metabolite with HPLC $R_t = 24.0$ min (50 mg). Analysis of the HRMS-EI spectrum of this metabolite suggested a molecular formula of $C_{13}H_{14}O_4$. The FTIR absorptions at 1795 and 1694 cm^{-1} suggested the presence of a carboxylic anhydride group. The ^1H NMR spectrum showed a broad singlet at δ 10.78 (D_2O exchangeable), three methyl singlets at δ 2.20–2.50 (likely attached to sp^2 carbons), and protons at δ 1.59 (d, $J = 8$ Hz) coupled to a methine proton at δ 4.18 (q, $J = 8$ Hz). The ^{13}C NMR data confirmed the presence of two carbonyl groups (δ 169.7, 166.7), five sp^3 carbons (δ 39.1, 22.7, 17.9, 14.9, 12.3) and six sp^2 carbons (δ 148.2 (s), 134.9 (s), 125.0 (s), 124.4 (s), 101.8 (s)), one of which was highly deshielded (δ 159.2) suggestive of a C−O linkage to aromatic ring. Analysis of the complete spectroscopic data of this phytotoxic metabolite allowed the assignment of the structure **1** (Figure 1). The FTIR and the ^1H NMR data of this compound matched that of sclerin (**1**) previously isolated from *S. sclerotiorum* (Satomura and Sato, 1963). Sclerin

(**1**) was isolated as a racemic mixture, not surprisingly considering that the proton at the only stereogenic centre of the molecule is epimerizable.

None of the extracts from the sclerotia of *S. sclerotiorum* showed phytotoxicity to *B. napus*, *B. juncea*, *S. alba*, or *E. gallicum* (Table 1). The toxicity of these extracts was determined using the brine shrimp lethality assay (Hostettmann, 1991). The results of these assays suggested that only the ethyl acetate extract was toxic to the brine shrimp larvae; all the shrimp larvae died in 24 hr when incubated with 1.0 mg/ml of EtOAc extract ($P < 0.001$); 78% of the larvae died when incubated with the same extract at a lower concentration of 0.1 mg/ml ($P = 0.003$). For the butanol extract, all the brine shrimp larvae survived in solutions even at concentrations of 1.0 mg/ml (Table 2). These results indicated that the butanol extracts of sclerotia did not contain substances toxic to brine shrimp and that most of the toxic substances present in the sclerotia of *S. sclerotiorum* were extracted with EtOAc.

The ethyl acetate extract of sclerotia of *S. sclerotiorum* was subjected to FCC, and all the fractions were assayed against brine shrimp larvae. Each of the toxic fractions obtained caused death to the brine shrimp larvae at concentrations as low as 0.1 mg/ml. However, at a concentration of 0.01 mg/ml, all the shrimp larvae survived. The toxic fractions were combined and purified by preparative

TABLE 2. TOXICITY OF EXTRACTS AND METABOLITES OF *Sclerotinia sclerotiorum* TO BRINE SHRIMP (*Artemia salina*) LARVAE

Extract/compound	Concentration	% Mortality[a] ± SD	P^b
Broth extract	1.0 (mg/ml)	0	$P > 0.05$
Sclerotia extract	1.0 (mg/ml)	100 ± 0	$P < 0.001$
	0.1 (mg/ml)	78 ± 5	$P = 0.003$
Fatty acid fraction	0.4 (mg/ml)	100 ± 0	$P < 0.001$
	0.1 (mg/ml)	90 ± 5	$P < 0.001$
	0.01 (mg/ml)	0	$P > 0.05$
Sclerin (**1**)	5×10^{-4} M	0	$P > 0.05$
	1×10^{-4} M	0	$P > 0.05$
Oleic acid	5×10^{-4} M	100 ± 0	$P < 0.001$
	1×10^{-4} M	10 ± 5	$P > 0.05$
Palmitic acid	5×10^{-4} M	5 ± 5	$P > 0.05$
	1×10^{-4} M	0	$P > 0.05$
Stearic acid	5×10^{-4} M	8 ± 5	$P > 0.05$
	1×10^{-4} M	0	$P > 0.05$

[a] % Mortality = 100 - [(number of live shrimp after 24 hr/number of live shrimp in control) × 100] ± standard deviation; results are the mean of three independent experiments conducted in triplicate.

[b] *P*-values denote the significant difference of the mean of number of live shrimp in saline water in control over number in saline water containing extract/compound in three independent experiments. Values were obtained using a one-tailed, paired-sample, *t*-test.

TLC. ^{1}H NMR analysis of the toxic fraction indicated it to be a mixture of fatty acids. After methylation of the fatty acid mixture with diazomethane, separation and analysis was achieved by GC-MS. Analyses of the EIMS data enabled the identification of three known fatty acids, namely hexadecanoic acid (palmitic acid, 22%), octadecanoic acid (stearic acid, 9%), and *cis*-9-octadecenoic acid (oleic acid, 42%). These structures were established unambiguously by comparison with authentic standards. These results are consistent with results of previous studies (Howell and Fergus, 1964), which determined that the major fatty acids of sclerotia from most fungi are the C-16 and C-18 acids. The remaining portion (27%) was determined to be composed of less common fatty acids of molecular formulae $C_{22}H_{40}O_2S$ (10%) and $C_{21}H_{38}O_2S$ (17%).

To test the phytotoxic effects of sclerin (**1**), oxalic acid, and broth and sclerotia extracts, different bioassays were developed using leaves of *B. napus*, *B. juncea*, *S. alba*, and *E. gallicum*. The results of phytotoxicity assays showed no significant differences between lesions observed on the scratched side of the leaf and lesions observed on the punctured side (Table 1). Sclerin (**1**) showed phytotoxic effects on *B. napus, B. juncea*, and *S. alba* causing necrotic and chlorotic tissue damage. Light brown lesions in circular patterns with an average diameter of about 3 mm were observed on the leaves of these crucifers when sclerin (5×10^{-4} M) or broth extract (1 mg/ml) were applied (Table 1). However, the sclerotium extracts (2 mg/ml) showed no toxicity to any of the species. The amount of sclerin (**1**) present in broth extracts indicated that sclerin (**1**) is the main component responsible for the phytotoxic activity of these extracts. It is also worth noting that sclerin (**1**) did not cause damage to the leaf tissues of *E. gallicum* at the tested concentrations. Importantly, oxalic acid even at a relatively high concentration (1×10^{-3} M) caused no macroscopic damage to any of the species, although it was reported to be a pathogenicity factor in *S. sclerotiorum* (Cessna et al., 2000) and to cause wilt damage to sunflower (*Helianthus annuus*) and other plant species (Hu et al., 2003). Nonetheless, the current studies cannot establish whether the differences among the various species are due to a lower sensitivity of brassicas to oxalic acid (see Table 3).

Using a leaf uptake bioassay, performed with four different concentrations of sclerin (**1**) and oxalic acid (1×10^{-3} to 1×10^{-5} M) in 2% acetonitrile, and broth extract (0.6 mg/1 ml of 2% acetonitrile), as described in the methods section, both sclerin (5×10^{-4} M) and broth extract of liquid cultures of *S. sclerotiorum* caused severe wilting of leaves (more intense in *S. alba*) after four days, whereas oxalic acid did not appear to have any effect. In the third type of assay, the spray bioassay, none of the solutions appeared to cause macroscopic damage to any of the plants.

The toxicity of extracts from cultures of *S. sclerotiorum* and compounds shown in Table 2 was determined using the brine shrimp lethality assay (Hostettmann, 1991). The ethyl acetate extract of sclerotia (1 mg/ml), fatty acid

TABLE 3. ELICITATION OF PHYTOALEXINS IN LEAF TISSUE OF *Erucastrum gallicum* USING MYCELIA OF *Sclerotinia sclerotiorum* AND $CuCl_2$ as Elicitors

Phytoalexin	Incubation time (hr)	Phytoalexins elicited by *S. sclerotiorum* (μmol/100 g of fresh leaf tissue)	Phytoalexins elicited by $CuCl_2$ (μmol/100 g of fresh leaf tissue)
Indole-3-acetonitrile (**7**)	24	0.54–0.63	Not detected
	48	0.12–0.33	0.58–1.08
	72	ND	0.89–1.92
	96	ND	1.89–2.22
Arvelexin (**8**)	24	0.79–1.35	Not detected
	48	0.23–0.44	2.54–4.40
	72	ND	1.21–1.96
	96	ND	1.04–1.82
1-Methoxy-spirobrassinin (**9**)	24	2.20–3.68	Not detected
	48	1. 05–1.51	2.03–2.74
	72	ND	2.66–3.03
	96	ND	1.55–3.03

Note. ND = not determined; leaf tissue disintegrates quickly after 48 hr incubation with *S. sclerotiorum*.

fraction (0.4 mg/ml), and oleic acid (5×10^{-4} M, the major component of the fatty acid fraction) caused 100% mortality to brine shrimp larvae ($P < 0.001$), while no significant toxic effect was observed for stearic acid or palmitic acid ($P > 0.05$; Table 2). These results indicate that oleic acid is responsible for the toxic effect of ethyl acetate extracts of sclerotia of *S. sclerotiorum*. Previous work indicated that the fatty acids present in *S. sclerotiorum*, *S. borealis*, and *Botrytis tulipae* (Sumner and Colotelo, 1970; Weete et al., 1970) varied if the fungi were isolated from host plants in the field or grown in the laboratory. Palmitic, oleic, and linoleic acids were the major free fatty acids of the laboratory-grown sclerotia, while a higher proportion of linoleic acid was found in sclerotia from natural sources (Weete et al., 1970). It is possible that the presence of the toxic oleic and linoleic acids in sclerotia has a protective/defensive role, e.g., preventing their consumption by animals, thus contributing to survival. Fatty acids isolated from *Aspergillis sydowi* have been reported to be toxic to brine shrimp (Curtis et al., 1974) and humans (Lima et al., 2002). Toxicity was found to increase with unsaturation in fatty acids, with oleic, linoleic, and linolenic acids being the most toxic. Fatty acids cause cell death via apoptosis or, when concentrations are greater, necrosis in man (Lima et al., 2002).

Phytoalexins from E. gallicum. A time-course response to biotic elicitation with mycelia of *S. sclerotiorum* as well as abiotic elicitation with $CuCl_2$, oxalic acid and sclerin (**1**) was investigated. The HPLC chromatograms of extracts of leaves elicited with *S. sclerotiorum* or $CuCl_2$ indicated the presence of three

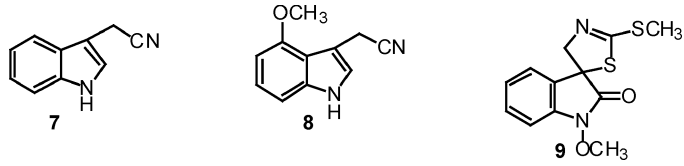

FIG. 2. Phytoalexins isolated from leaves of *Erucastrum gallicum*: indole-3-acetonitrile (7), arvelexin (8), and methoxyspirobrassinin (9).

compounds with $R_t = 11.2$ min., 13.1 min., and 16.9 min, respectively, that were not present in control leaves. Sclerin (**1**) and oxalic acid did not seem to elicit any of these compounds even when plants were incubated up to 7 days. Elicitation of phytoalexins by *S. sclerotiorum* was substantially faster (ca. 24 hr vs. 48 - 96 hr) than that observed for $CuCl_2$.

The compounds with $R_t = 11.2$ min. and 13.1 min were identified as indole-3-acetonitrile (**7**), and arvelexin (**8**), (Figure 2) by comparison with authentic synthetic samples available in our laboratory. In order to obtain reasonable amounts of extract to isolate and determine the structure of the compound responsible for the peak at $R_t = 16.9$ min (HPLC), a larger amount of $CuCl_2$ elicited leaves were processed in a manner similar to that used in the time-course study to give 1-methoxyspirobrassinin (**9**, 5.0 mg, $R_t = 16.9$ min). The HRMS data for the metabolite with $R_t = 16.9$ min (**9**) (Figure 2) indicated a molecular formula of $C_{12}H_{12}N_2S_2O_2$ (obtained m/z 280.0336, calcd. 280.0340), further corroborated by analysis of the NMR spectroscopic data. The NMR spectra of this compound displayed resonances in the aromatic region indicative of a 2-oxindole moiety as well as signals due to methoxy (δ_H 3.99; δ_C 63.9), and thiomethyl groups (δ_H 2.62; δ_C 15.3), a carbonyl or equivalent group (δ_C 163.2), and a methylene group (δ_H 4.60, d, $J = 15.5$ Hz; δ_H 4.46, d, $J = 15.5$ Hz; δ_C 72.6). The ^{1}H NMR, FTIR, and MS-EI spectral data for metabolite **9** were identical to that of 1-methoxyspirobrassinin, a phytoalexin isolated from *Brassica oleracea* (Gross et al., 1994). However, the ^{13}C NMR data of 1-methoxyspirobrassinin is reported here for the first time.The antifungal activity of 1-methoxyspirobrassinin (**9**) and arvelexin (**8**) was determined using the mycelia radial growth bioassay described in the methods. These antifungal bioassays established that 1-methoxyspirobrassinin (**9**) and arvelexin (**8**) were active against *S. sclerotiorum* and *R. solani* (root rot fungus), two of the most important pathogens of oilseed and vegetable crucifers. 1-Methoxyspirobrassinin (**9**, 5×10^{-4} M) caused 68% inhibition to *R. solani* and 53% to *S. sclerotiorum* after 72 hr of incubation, whereas arvelexin (**8**, 5×10^{-4} M) showed 66% inhibition to *R. solani* and 46% to *S. sclerotiorum* after 72 hr (Table 4, $P < 0.001$). Both indole-3-acetonitrile (**7**), isolated from elicited leaves of *B. juncea* (Pedras et al., 2002), and arvelexin (**8**), isolated from elicited leaves of *Thlaspi arvense* (Pedras

TABLE 4. ANTIFUNGAL ACTIVITY OF ARVELEXIN (**8**) AND 1-METHOXYSPIROBRASSININ (**9**) AGAINST *Rhizoctonia solani* AND *Sclerotinia sclerotiorum*

Compound	Concentration	% Inhibition *R. solani*[a,b]	% Inhibition[a] *S. sclerotiorum*[a,b]
Arvelexin (**8**)[c]	5×10^{-4} M	66***	46***
	2.5×10^{-4} M	36***	10***
	5×10^{-5} M	0*	0*
1-Methoxy spirobrassinin (**9**)	5×10^{-4} M	68***	53***
	2.5×10^{-4} M	14***	34***
	5×10^{-5} M	0*	10***

[a] Three days of incubation under constant light; percent inhibition = 100—[(growth on medium containing compound/growth on control medium) × 100]; results are the mean of three independent experiments conducted in triplicate. Error associated with determining zones of inhibition is ±0.5 mm.
[b] *P* values denote the significant difference of the mean of diameter of radial growth of mycelia (mm) in medium containing compound over growth in control medium in three independent experiments. *** $P < 0.001$; * $P > 0.05$. Values obtained using a one-tailed, paired-sample *t*-test.
[c] Synthetic sample (Pedras et al., 2003).

et al., 2003), a wild crucifer resistant to blackleg disease were reported as phytoalexins strongly active against the blackleg fungus (*Leptosphaeria maculans*, asexual stage *Phoma lingam*).

In summary we have established for the first time that *Sclerotinia sclerotiorum* produces a somewhat selective phytotoxin that is phytotoxic to three cruciferous species susceptible to Sclerotinia stem rot disease but not to a resistant species (*E. gallicum*). Sclerin (**1**, 5×10^{-4} M) caused severe necrosis and chlorosis to leaves of *B. napus*, *B. juncea*, and *S. alba*. On the other hand, oxalic acid showed no phytotoxicity to any of the species tested, only superficial leaf damage was observed when oxalic acid was applied at a rather high concentration (5×10^{-2} M). One could argue that at such a high concentration many substances would show toxicity to plants. Neither oxalic acid nor sclerin (**1**) were detected in infected leaves of *E. gallicum*. In addition, we have shown that oleic acid, the major fatty acid isolated from sclerotia of *S. sclerotiorum* is responsible for the toxic activity of extracts of sclerotia to brine shrimp larvae (*A. salina*). However, sclerin (**1**), the broth extract, and oxalic acid showed no toxicity to brine shrimp larvae.

Phytoalexin elicitation in leaves of *E. gallicum* (resistant to *S. sclerotiorum*) led to the isolation of three known phytoalexins: indole-3-acetonitrile (**7**), arvelexin (**8**), and 1-methoxyspirobrassinin (**9**). (Figure 2) Both arvelexin (**8**) and 1-methoxyspirobrassinin (**9**) showed antifungal activity against *S. sclerotiorum* and *R. solani*. To the best of our knowledge, this is the first time that these

phytoalexins are reported to be produced by the wild crucifer *E. gallicum*. Considering that resistance of *E. gallicum* to *S. sclerotiorum* is potentially transferable to *B. rapa*, a susceptible canola species, and that phytoalexins **8** and **9** are not produced by *B. rapa* (Pedras et al., 2000b), arvelexin (**8**) and 1-methoxyspirobrassinin (**9**) together with the lack of response to sclerin (**1**) may become useful markers for resistance to *S. sclerotiorum* in hybridized lines of *B. rapa* × *E. gallicum*.

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THE ALLELOCHEMICAL SORGOLEONE INHIBITS ROOT H^+-ATPase AND WATER UPTAKE

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Abstract—Sorghum plants inhibit the growth of some adjacent species. Root exudates from grain sorghum (*Sorghum bicolor*), consisting primarily of the quinone sorgoleone, are phytotoxic to several plant species, yet the mechanisms of growth inhibition remain to be fully explained. Disruption of electron transport functions in isolated mitochondria and chloroplasts has been reported as one explanation for growth inhibition. In the studies reported here, however, soybean seedlings grown in nutrient solution with 10, 50, or 100 μM sorgoleone showed no disruption of photosynthesis, as measured by leaf fluorescence and oxygen evolution, yet their mean leaf surface area was less when grown in 100 μM sorgoleone. Furthermore, in the presence of these same concentrations of sorgoleone, decreased nutrient solution use by soybean seedlings and decreased H^+-ATPase activity in corn root microsomal membranes were observed. This suggests that impairment of essential plant processes, such as solute and water uptake, driven by proton-pumping across the root cell plasmalemma should also be considered as a mechanism contributing to observed plant growth inhibition by sorgoleone.

Key Words—Sorgoleone, *Glycine max, Zea mays, Sorghum bicolor,* H^+-ATPase, water uptake, sorghum root exudate, allelopathy, photosynthesis, respiration.

INTRODUCTION

Many *Sorghum* spp. are used in agricultural areas of the United States as a cover crop because of a reputation for weed suppression. Numerous laboratory and field studies showing allelopathic interference support this (Breazeale, 1924; Hussain and Gadoon, 1981; Lehle and Putnam, 1983; Putnam et al., 1983;

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Panasiuk et al., 1986; Einhellig and Rasmussen, 1989; Weston et al., 1989; Einhellig and Souza, 1992). This suppression may last even into the following growing season as a result of sorghum residues (Einhellig and Rasmussen, 1989). Several bioassays using broadleaf and grass weed species have shown a concentration-dependent suppression of root elongation and seedling growth, as well as interference with the cell cycle, by a hydrophobic quinone, sorgoleone, exuded from the root hairs of grain sorghum (Netzly and Butler, 1986; Einhellig and Souza, 1992; Nimbal et al., 1996a; Hallak et al., 1999). These phytotoxic properties suggest that sorgoleone, shown to consist primarily of 2-hydroxy-5-methoxy-*e*-[(8*Z*,11*Z*)-8,11,14-pentadecatriene]-*p*-benzoquinone (Netzly and Butler, 1986), may be a factor in suppressive effects associated with *Sorghum* spp.

We have previously shown inhibitory effects of sorgoleone on respiration and photosynthesis *in vitro*, providing evidence that perturbations in energy transformations may be a mechanism for sorgoleone's phytotoxicity (Rasmussen et al., 1992; Einhellig et al., 1993). Nimbal et al. (1996a) also demonstrated that sorgoleone inhibits O_2 evolution and identified photosystem II (PSII) as the site of action. Subsequent studies revealed that sorgoleone blocks electron transport by binding to the Q_B binding site of PSII, and it was suggested that this is a primary mode of action (Nimbal et al., 1996a,b; Gonzalez et al., 1997; Czarnota et al., 2001). Kagan et al. (2003) isolated sorgoleone congeners and showed that these minor analogues also inhibit photosynthetic O_2 evolution *in vitro*. However, it remains to be established that sorgoleone is actually translocated to thylakoids *in vivo,* and it is probable that it has multiple sites of action. Meazza et al. (2002) showed that sorgoleone inhibits hydroxyphenylpyruvate dioxygenase (HPPD) *in vitro*. This enzyme is necessary for synthesis of carotenoids, including the precursor of plastoquinone, thus, its effect on PSII *in vivo* may be both direct and indirect (Inderjit and Duke, 2003).

Fedtke (1993) argued that many hydrophobic compounds have little activity beyond the root. Perturbations of root metabolism can significantly impair plant growth and may explain the activity of some phytotoxins. For example, we recently reported that juglone, a quinone exuded by the roots of *Juglans* spp., inhibited root H^+-ATPase activity and caused decreased water uptake in hydroponically grown corn and soybean seedlings (Hejl and Koster, 2004).

Here, we report that, like juglone, sorgoleone also can perturb root metabolism before significant effects on photosynthesis are seen. In these studies, chlorophyll fluorescence and oxygen evolution by leaves of hydroponically grown soybean seedlings were measured to examine how sorgoleone affects photosynthesis *in vivo*. Nutrient solution uptake was assessed to evaluate whether this root function was affected by the presence of the sorghum exudate. Finally, the activity of plasma membrane H^+-ATPase in corn root microsomes was evaluated during exposure to sorgoleone to determine whether inhibition by the phytotoxin may also be a mode of action.

METHODS AND MATERIALS

Sorgoleone Collection and Purification. Sorghum root exudate was collected from the roots of grain sorghum seedlings [*Sorghum bicolor* (L.) Moench., Dekalb Hybrid DK 28 or Den Besten DB-130] according to a previously published method (Netzly et al., 1988; Einhellig and Souza, 1992). Before it was evaporated to dryness, the amber-colored extract in methylene chloride was filtered through Whatman no. 1 filter paper to remove small particulate matter. The dark brown, tar-like exudate was stored at -4°C until use, and was used within 6 months of extraction.

Soybean Growth in Hydroponics. Soybean seeds (*Glycine max* L. Merr., Prairie Brand 277) were germinated, and growth experiments were conducted under greenhouse conditions in Vermillion, SD, USA, in July with night temperatures between 20 and 25°C and daytime temperatures up to 30–35°C. Five- to 6-day-old seedlings were selected for size uniformity and transferred to individual opaque growth vials containing 70 ml of nutrient solution according to Hejl and Koster (2004), modified from Einhellig and Souza (1992). After 3-days acclimation, 10 replicate random seedlings for each treatment group were provided with fresh nutrient solution amended with 10, 50, or 100 μM sorgoleone. Since sorgoleone has low solubility in water, it was delivered into the nutrient solution as a concentrated stock solution in methanol [final concentration of 1% (v/v)] as previously described (Netzly et al., 1988; Fedtke, 1993; Nimbal et al., 1996a). Control treatments with and without methanol were also tested. Every 2–3 days, each plant was provided fresh nutrient solution with the same amendment to ensure that sufficient amounts of ions, unoxidized iron, and water were available.

Chlorophyll Fluorescence and Leaf CO_2-Dependent Oxygen Evolution. To assess the effect of sorgoleone on photosynthesis *in vivo*, chlorophyll fluorescence was measured every 2–3 days immediately before nutrient solution replacement and after plants were exposed to at least 2 hr of light. The two most fully emerged leaves on each plant were selected and dark-adapted for 15 min. Initial fluorescence (F_0) and maximal fluorescence (F_m) were measured using an Opti-Sciences OS-100 modulated fluorometer (PP Systems, Inc. USA). The efficiency of excitation capture was recorded as F_v/F_m and calculated on the fluorometer, which used the formula $F_v = F_m - F_0$ to determine variable fluorescence (F_v) (Hejl and Koster, 2004).

Oxygen evolution in soybean seedlings exposed to sorgoleone was also evaluated. Seeds were germinated 5–6 days in vermiculite under greenhouse conditions, and seedlings were selected for uniformity in size and transferred to opaque vials containing 70 ml of nutrient solution. After 2-days acclimation, seedlings were transferred to fresh nutrient solution amended with either 0 or 100 μM sorgoleone and returned to the same growth conditions. To avoid confounding results with methanol, aliquots of the sorghum root exudate were measured into individual

vials of the heated nutrient solution. However, with this method, delivery of concentrations below 100 μM could not be accomplished with confidence. After 7-days treatment, the most recent fully emerged leaf of each plant was harvested, and leaf areas were determined. Oxygen evolution was measured at 25°C in an air-phase chamber fixed to a Hansatech Clark-type O_2 electrode (Kings Lynn, Norfolk, England) according to a procedure modified from Delieu and Walker (1981) and Hejl and Koster (2004). Each leaf section was laid on a mesh wire disk resting on a sponge soaked with 500 μl of a 1 M $NaHCO_3$ solution as a source of CO_2. The chamber was sealed and calibrated (nmol O_2 per mV). After a linear rate was established, mV change was recorded for 10–15 min, and O_2 evolution was calculated from the most linear 5-min section of the recorded slope and reported as nmol O_2 cm^{-2} min^{-1}.

Nutrient Solution Use. To confirm initial observations that sorgoleone-treated seedlings take up less nutrient solution, the volume of the unused solution was measured for each group at each time of solution change. This volume was used to calculate mean solution uptake per seedling.

Isolation of Corn Root Membranes and ATPase Assay. Corn seeds (*Zea mays* L., Cargill 6327F14) were germinated, and the microsomal fraction was obtained according to a procedure described by Hejl and Koster (2004). H^+-ATPase activity by microsomal membranes was assayed as previously described (Hejl and Koster, 2004) using replicates amended with 0, 5, 10, 50, 100, 250, 500, or 1000 μM sorgoleone dissolved in ethanol. Inorganic phosphate (P_i) release was determined colorimetrically at 700 nm against a water blank, and reported as percent of control. Because sorgoleone is an amber-colored compound, experimental trials with only sorgoleone were done to determine if the compound's color contributed to the absorbance at 700 nm. Sorgoleone did cause a small, but statistically significant, amount of absorbance at concentrations of 500 and 1000 μM. Therefore, the mean absorbance of the sorgoleone controls at these concentrations was subtracted from the experimental data to correct mathematically for the contribution of sorgoleone to the absorbance.

Statistics. Leaf area and oxygen evolution data were subjected to a *t*-test to determine significant differences between means ($P < 0.05$). Nutrient solution use data were subjected to one-way ANOVA ($P < 0.05$) with significant differences identified by Student-Newman-Keuls test. Data from leaf fluorescence studies were subjected to two-way ANOVA with treatment and days as predictor variables. Means comparisons were adjusted to reduce Type I error rate with Bonferroni type adjustment at 0.008 level of probability (Zar, 1996).

RESULTS AND DISCUSSION

Mean leaf area was significantly smaller in hydroponically grown soybean plants treated with 100 μM sorgoleone compared to controls (Table 1), and

TABLE 1. LEAF SURFACE AREA AND OXYGEN EVOLUTION FOR SOYBEANS EXPOSED TO 0 AND 100 μM SORGOLEONE FOR 7 DAYS[a]

Sorgoleone (μM)	Leaf surface area (cm^2)	Oxygen evolution (μmol O_2 cm^{-2} hr^{-1})
0	6.2 ± 1.3 a	13.6 ± 1.6 a
100	4.2 ± 1.3 b	14.4 ± 4.7 a

[a] Means within each experiment followed by different letters were significantly different at $P < 0.05$ (t-test; $N = 8$–9).

seedlings in the 50 and 100 μM sorgoleone treatment groups appeared smaller. Many studies have previously reported concentration-dependent inhibition of growth of various species in the presence of sorgoleone (Einhellig and Souza, 1992; Nimbal et al., 1996a; Czarnota et al., 2001; Weston and Czarnota, 2001). However, these studies also report considerable differential tolerance to sorgoleone by the various species tested.

Roots of soybean seedlings exposed to sorgoleone showed slight brown discoloration and appeared to have decreased length and lateral branching. In contrast, Netzly and Butler (1986) found that elongation of roots in germinating corn seedlings was not affected by sorgoleone. Nimbal et al. (1996a) similarly reported root discoloration in the presence of sorgoleone in several hydroponically grown weed species, but root weights were generally less affected by the presence of sorgoleone than shoot weights. Weston and Czarnota (2001) also reported that, after a 3-wk growth period in soil impregnated with sorgoleone at concentrations ranging from 10 to 80 ppmw (parts per million by weight), root development in three broadleaf species exhibited little or no effect; however, a significant, concentration-dependent inhibition of shoot growth had occurred. Grass species tested in the same study showed little effect on the growth of either roots or shoots. Research is needed to clarify the observed differential effects among species and on root and shoot growth by sorgoleone.

Since sorgoleone at concentrations as low as 0.2 μM impairs respiration and photosynthesis in isolated mitochondria and chloroplasts (Rasmussen et al., 1992; Einhellig et al., 1993), we expected that oxygen evolution and chlorophyll fluorescence data would show impairment of photosynthesis *in vivo*. However, oxygen evolution from leaves of sorgoleone-treated seedlings did not differ from values obtained for untreated control plants (Table 1). Chlorophyll fluorescence parameters showed some small increase in F_v/F_m ratios in the initial days of treatment; however, they did not differ significantly from methanol-only treatments, and none of the treatments showed consistent significant differences after 9 d (data not shown). An increase in F_v/F_m value would indicate increasing inhibition of PSII reaction centers (Petersen et al., 1988; Gleiter and Renger, 1993), as reported in a number of studies using isolated thylakoid membranes (Nimbal et al., 1996a,b; Gonzalez et al., 1997; Czarnota et al., 2001).

Sorgoleone bears a structural resemblance to plastoquinones and ubiquinones (Czarnota et al., 2001), essential carriers in electron transport reactions of photosynthesis and respiration, making competitive inhibition of electron transport in both respiration and photosynthesis a theoretical consideration. Studies reporting inhibition *in vitro*, suggest that disruption of photosynthesis and respiration may be a primary mode of action for sorgoleone. However, the lack of significant differences between controls and treatments in leaf oxygen evolution (Table 1) and F_v/F_m ratios *in vivo* raises questions as to whether disruption of photosynthesis is a primary or sole mechanism of growth inhibition mediated by sorgoleone, at least for soybean. It has been shown previously in hydroponic bioassays of plant growth that different species demonstrate differential tolerance to sorgoleone exposure (Einhellig and Souza, 1992; Nimbal et al., 1996a). Further studies to reveal whether some species compensate physiologically to overcome the effects of sorgoleone on photosynthesis or whether they avoid transport of the phytotoxin to the chloroplasts are crucial to understanding if sorgoleone directly affects photosynthesis *in vivo*.

The problem of delivering sorgoleone in accurate concentrations into nutrient solution via an agent that itself had no effect also confounds interpretation of these results. Fedtke (1993) reported using methanol to dissolve herbicides for delivery into rooting bioassays. Netzly et al. (1988) and Nimbal et al. (1996a) both reported using methanol to deliver sorgoleone to filter paper in seed germination bioassays. In our bioassays, however, we found that methanol had a significant effect itself, making it difficult to interpret the effects of sorgoleone when it was delivered to nutrient solution via methanol. Nimbal et al. (1996a) also delivered sorgoleone via acetone, but gave no results of acetone controls. In our oxygen evolution study, sorgoleone was delivered directly into heated solution in each vial, which increased the solubility of the exudates; however, concentrations less than 100 μM could not be delivered accurately.

Solution uptake by seedlings was significantly reduced at the 100 μM sorgoleone treatment level compared to both the unamended controls and controls amended with only methanol (Table 2). Seedlings treated with 10 and 50 μM sorgoleone also showed decreased solution use, but were not statistically different in average daily use from methanol controls (Table 2). Although interpretation of the results of nutrient solution use was confounded somewhat by the effects of methanol, the significant decrease with the 100 μM treatment suggests that impaired water uptake and the multiple physiological consequences arising from water deficit should be considered among the mechanisms of growth inhibition by sorgoleone.

An underlying cause of diminished solution uptake may be found in the effects of sorgoleone on plasma membrane H^+-ATPase activity. Decreased H^+-ATPase activity in root microsomal membranes was apparent at 5 μM sorgoleone and was statistically significant at concentrations of 10 μM and above (Figure 1).

TABLE 2. SOYBEAN AVERAGE DAILY NUTRIENT SOLUTION USE AFTER 16-DAYS TREATMENT WITH SORGOLEONE[a]

Sorgoleone (μM)	Average daily nutrient solution use (ml) per seedling
Control	14 ± 3 a
Methanol	12 ± 3 b
10	11 ± 3 b
50	12 ± 2 b
100	8 ± 2 c

[a] Means within each experiment followed by different letters were significantly different at $P < 0.05$ (one-way ANOVA, Student-Newman-Keuls test; $N = 18$).

Because inhibitors of tonoplast and mitochondrial ATPases and phosphatase were present, the measured phosphate release should be largely from plasma membrane H^+-ATPase. However, concentrations of up to 1000 μM sorgoleone, even after mathematical correction for the color contribution of sorgoleone, did not inhibit P_i release equal to 200 μM sodium vanadate, a known inhibitor of the plasma membrane H^+-ATPase (Calera et al., 1995). In combination, 250 μM sorgoleone and 200 μM sodium vanadate impaired H^+-ATPase activity only slightly, but significantly more than sodium vanadate alone. The inhibition by sorgoleone reported here was similar to that caused by juglone, another naturally occurring quinone exuded from the roots of black walnut (*Juglans nigra* L.) and related *Juglans* spp. (Hejl and Koster, 2004).

Inhibition of plasma membrane H^+-ATPase not only can explain decreased water uptake by seedlings exposed to sorgoleone, but offers explanation, at least in part, for documented growth impairment attributed to sorghum root exudate. Proper functioning of the plasma membrane H^+-ATPase enzyme is essential to maintaining a high proton concentration in the rhizosphere immediately external to root cell membranes. This produces an electrochemical gradient driving the uptake of essential ions and other solutes, which, in turn, drives water uptake (Briskin and Hanson, 1992; Michelet and Boutry, 1995; Steudle and Peterson, 1998). Such a fundamental disruption of root function could impose indirect disturbances on many other essential plant functions, including photosynthesis and respiration, without the phytotoxin ever reaching the tissues and organelles where these reactions occur.

A number of studies have reported allelochemical-induced disruption of plant water balance and perturbations in ion uptake in roots, and some suggest that this is due to interference with normal membrane function and disruption of active transport (Glass and Dunlap, 1974; Barkosky et al., 1999, 2000). Barkosky et al. (1999) conclude that disruptions in water relations appear to be the primary mode

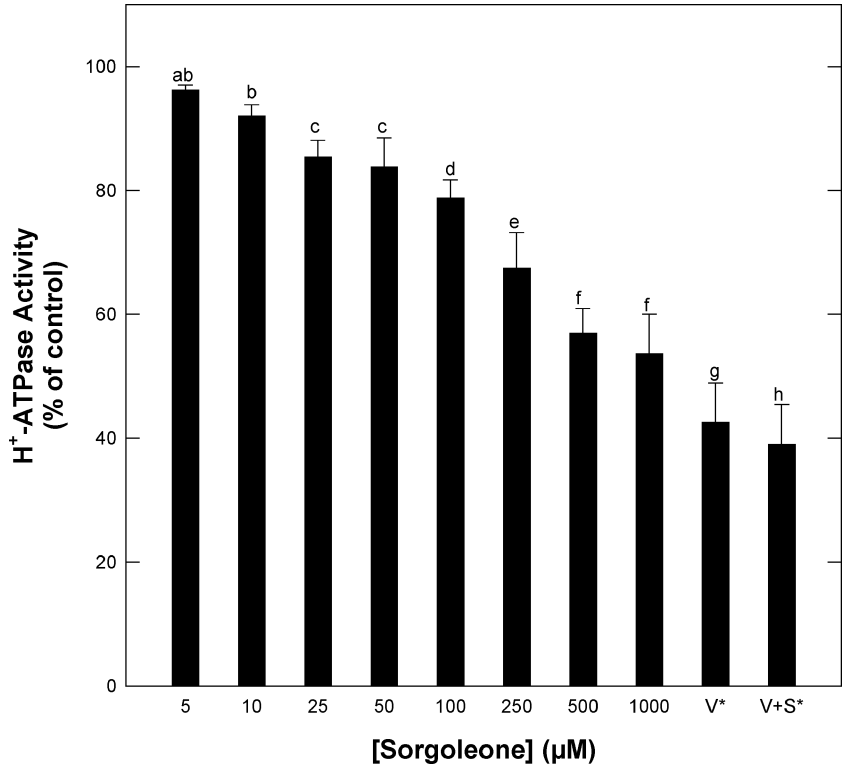

FIG. 1. Influence of sorghum root exudate on H^+-ATPase activity in corn root microsomal fractions Error bars represent one standard error of the mean. Bars with different letters are significantly different at $P < 0.05$ (Student-Newman-Keuls test, $N = 5$–40). *V = 200 μM sodium vanadate; V + S = 200 μM sodium vanadate + 250 μM sorgoleone.

of action that leads to overall reduction in growth in leafy spurge in the presence of the phytotoxin hydroquinone. Inhibition of enzyme activity is a fairly common mode of herbicide action (Einhellig, 1995), and some studies have reported herbicides to be inhibitors of plasma membrane H^+-ATPase (Tu et al., 1995; Hull and Cobb, 1998). A number of other substances inhibit plasma membrane H^+-ATPase activity (Blein et al., 1988; Che et al., 1992; Di Giorgio et al., 1994; Friebe et al., 1997; Hejl and Koster, 2004).

Another possible mechanism that merits investigation is interference with plasma membrane redox systems. Sorgoleone, like many quinones, is a strong electron acceptor, so it may be able to interrupt electron transport by plasma membrane redox systems, much as it does the electron transport chains of mitochondria (Rasmussen et al., 1992) and thylakoids (Nimbal et al., 1996b). Plant plasma

membrane redox systems transfer electrons to extracellular electron acceptors and are believed to be involved in elongation growth, cell division, apoplastic radical production, and generation of membrane potential (Crane and Møller, 1988; Morré et al., 1988; Lüthje et al., 1998). Sorgoleone growth inhibition could be a reasonable consequence of disruptions to plasma membrane redox systems; however, to our knowledge this has not been studied.

Our results support the hypothesis that sorgoleone disturbance of plasma membrane H^+-ATPase activity in root cells may explain growth interference in the presence of sorghum root exudate. They also raise questions as to whether direct interference with photosynthesis is a major or sole mechanism, even though other studies have shown sorgoleone to be a potent inhibitor of both photosynthesis and respiration *in vitro* (Rasmussen et al., 1992; Einhellig et al., 1993; Gonzalez et al., 1997). It is possible that allelopathic perturbations of root function could affect plant growth and indirectly affect energy transformation processes, even though the inhibitor never reaches the mitochondria or chloroplasts.

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PLANT–PLANT SIGNALING: APPLICATION OF *trans*- OR *cis*-METHYL JASMONATE EQUIVALENT TO SAGEBRUSH RELEASES DOES NOT ELICIT DIRECT DEFENSES IN NATIVE TOBACCO

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Abstract—*Nicotiana attenuata* plants growing in close proximity to damaged sagebrush (*Artemisia tridentata* ssp. *tridentata*) suffer less herbivory than plants near undamaged sagebrush. Sagebrush constitutively releases methyl jasmonate (MeJA), a compound that when applied directly to *N. attenuata*, elicits herbivore resistance and the direct defense traits [protease inhibitors (PIs), nicotine]. Damage increases the release of volatile MeJA, primarily in the cis epimer, suggesting that *cis*-MeJA may mediate this apparent interplant signaling. We characterized sagebrush's MeJA plume before and after damage in nature and in the laboratory, and compared the activity of *trans*- and *cis*-MeJA in inducing PIs, nicotine, and *Manduca sexta* resistance in *N. attenuata*. We used both lanolin applications and aqueous sprays that mimic natural exposures, and we determined the amount of volatilized MeJA required to elicit a nicotine response in open-grown plants. Wounding rapidly and transiently increased *cis*-MeJA emissions from damaged parts (but not systemically), and the released plume did not rapidly dissipate in nature. *cis*-MeJA was not consistently more active than *trans*-MeJA, and the order of exposure (*trans*- then *cis*-) did not influence activity. We conclude that volatile MeJA, either *trans*- or *cis*-, when applied at

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levels consistent with those released by sagebrush does not elicit direct defenses in *N. attenuata*.

Key Words—*Nicotiana attenuata*, *Artemisia tridentata* ssp. *tridentata*, *cis*-MeJA, *trans*-MeJA, interplant communication, induced defenses, protease inhibitor, nicotine, *Manduca sexta*.

INTRODUCTION

Interplant communication *via* airborne signals has received much attention recently (Karban and Baldwin, 1997; Dicke and Bruin, 2001; Dicke et al., 2003). While some studies have examined only interactions within laboratory bell jars (Farmer and Ryan, 1990; Shulaev et al., 1997; Preston et al., 1999; Arimura et al., 2000a,b), two studies provide evidence for communication in natural populations. In populations of alder (*Alnus glutinosa*), herbivory experienced by undamaged alders was inversely related to their distance from an artificially damaged tree, and subsequent laboratory experiments demonstrated that leaves collected from undamaged trees growing closer to the damaged tree were less desirable to herbivores for both consumption and oviposition compared to leaves taken from more distant trees (Dolch and Tscharntke, 2000). Either airborne or soilborne signals may be responsible for the observed change in resistance. In a second study, wild tobacco (*Nicotiana attenuata*) plants growing adjacent to damaged sagebrush (*Artemisia tridentata* ssp. *tridentata*) plants suffered less herbivory than tobacco plants located near undamaged sagebrush, an interaction that appears to be mediated by an airborne signal (Karban et al., 2000). The signal(s) mediating this interaction has not yet been identified.

Sagebrush is an aromatic plant that releases a complex blend of volatiles (Muller et al., 1966; Kelsey et al., 1978; Personius et al., 1987; Preston et al., 2001). Included in this volatile blend is the methyl ester of a ubiquitous plant hormone, jasmonic acid (JA). While a number of volatiles are released at greater levels after damage than methyl jasmonate (MeJA; Preston et al., 2001), MeJA is a likely candidate for the signal mediating the interplant communication because of its activity in eliciting herbivore resistance in *N. attenuata* (Baldwin, 1998) and protease inhibitors (PIs) in tomato (Farmer and Ryan, 1990). Exposure of *N. attenuata* to volatile MeJA differentially regulates several genes, some believed responsible for orchestrating complex metabolic shifts after herbivore attack (Hermsmeier et al., 2001). Moreover, these changes in transcript accumulation are likely linked to many of the well-studied JA-induced responses in *N. attenuata*, including changes in levels of nicotine, phenolics, flavonoids, phenolic putrescine conjugates, PIs, PPOs, diterpene sugar esters, and volatile releases of monoterpenes, sesquiterpenes, C_6 alcohols, and aldehydes (Baldwin et al., 1998; Halitschke et al., 2000; Kahl et al., 2000; Keinänen et al., 2001; van Dam et al., 2001).

Two fundamental predictions for an effective airborne signal are that (1) the signal must be closely associated with damage, with quantitative or qualitative changes providing information, and (2) it must reach the recipient at physiologically active levels (Firn and Jones, 1995). The MeJA released by damaged sagebrush exhibits both quantitative and qualitative changes as the amount of MeJA released increases, due primarily to an increase in the amount of a single epimer, *cis*-MeJA (Karban et al., 2000). This epimeric change is interesting as the cis orientation is thermodynamically less stable, rapidly epimerizing to the more stable trans configuration, until an equilibrium trans–cis ratio of approximately 93:7 is attained (Beale and Ward, 1998), but it is thought to be the biologically more active of the two naturally occurring epimers of MeJA (Beale and Ward, 1998; Sarkar and Ghorai, 1999). Alternatively, the activity of the airborne signal may not be due to the absolute amounts of *cis*- and *trans*-MeJA released after damage, but perhaps the change in the predominate epimer. *Nicotiana sylvestris*, a tobacco species native to Central America, exhibits an immunological "memory" of previous MeJA exposures that is readily seen as a more rapid increase in nicotine concentrations of previously exposed but uninduced plants compared to uninduced plants without previous MeJA exposures (Baldwin and Schmelz, 1996). This immunological memory suggests that those plants that experience low levels of *trans*-MeJA (i.e. from an undamaged sagebrush plant) may become sensitized and respond rapidly when exposed to *cis*-MeJA released from a damaged sagebrush plant. Similarly, corn (*Zea mays*) seedlings exposed to green leaf volatiles from herbivore-attacked neighboring plants respond to damage with greater JA and volatile sesquiterpene levels than seedlings without prior exposure (Engelberth et al., 2004).

Sagebrush's MeJA emission has only been characterized from excised branches in bell jars (Farmer and Ryan, 1990), and from undamaged sagebrush plants and for 1–2 hr immediately following damage to plants growing in the field (Karban et al., 2000). However, the longevity of the increased cis emission after damage, whether the release is systemic (whole plant) or localized to damaged tissues, and the distance over which the signal is detectable are unknown. Other volatile organic compounds (VOCs) released from damaged plants are known to exhibit temporal and spatial variability (Paré and Tumlinson, 1999; Halitschke et al., 2000). Immediately after damage, VOC releases occur local to the damaged area and consist primarily of "green leaf" volatiles (mainly C_6 aldehydes, alcohols, and acetates) that are followed by a delayed (2–24 hr) systemic release of other compounds. The release of these VOCs, which are triggered by herbivore-specific elicitors, can function as indirect defenses by attracting predators and parasitoids to feeding herbivores (Röse et al., 1996; De Moraes et al., 1998, 2001; Turlings et al., 1998; Paré and Tumlinson, 1999; Keßler and Baldwin, 2001).

Here, we characterize both the emission of MeJA and the response of *N. attenuata* to exposures of *trans*- and *cis*-MeJA in ecologically relevant quantities. We quantify the amount and trans–cis ratio of MeJA released by *Artemisia*

tridentata ssp. *tridentata* by trapping volatiles from natural populations in the Great Basin Desert of southwestern Utah before and after damage. After damage, we determine: (1) the kinetic of the release; (2) if the release is local to the damaged tissue or also from undamaged leaves; and (3) the distance over which the MeJA release is detectable. We determine the concentration of JA and MeJA in sagebrush leaves to determine the size of the endogenous pools for MeJA emission. Second, we compare the activity of *cis*- and *trans*-MeJA in laboratory experiments using two methods of delivering the MeJA to the plant: in lanolin applications and aqueous sprays. Lanolin application quantitatively delivers MeJA to a defined area of leaf. Aqueous sprays deliver MeJA as an aerosol over the plant canopy, thereby simulating volatile exposure. We test the activity of both trans and cis epimer by measuring two known JA-induced defense metabolites, namely nicotine and protease inhibitors (PIs), as well as monitor the short-term growth of an herbivore, *Manduca sexta*, that commonly attacks *N. attenuata* in nature (Keßler and Baldwin, 2001) and against which MeJA applications elicit resistance in the laboratory (van Dam et al., 2000). We tested the epimers individually and in a sequence mimicking the exposures that occur when *N. attenuata* grows immediately adjacent to an undamaged sagebrush that is subsequently damaged.

METHODS AND MATERIALS

Volatile Collection. Volatile collection and analysis followed methods described in Karban et al. (2000) and Preston et al. (2001). Briefly, sagebrush volatiles were collected by pulling air through traps containing activated charcoal (150-mg ORBO traps; Supelco, Bellefonte, PA) for 2–8 hr, depending on the experiment. The flow rate of air through the traps was 450–500 ml/min (measured by a mass flow meter: Aalborg Instruments, Orangeburg, NY). With the exception of the experiment that determined the distance over which MeJA is detected from sagebrush, branches were enclosed in 3.75-l transparent plastic conical containers, with an open bottom (22-cm i.d.) and top (7-cm i.d.) such that the volume of the headspace was equal for all trappings. For determining the distance over which MeJA is detectable, no sampling containers were used so as not to disturb the dispersion of volatiles from the sagebrush. All traps were stored at -20°C before being transported to the laboratory on dry ice where they were again stored at -20°C until analysis. After trapping and prior to elution, 710 ng of the internal standard, a triple ^{13}C-labeled MeJA (MW = 227) with a trans–cis ratio of 96:4, was added to each trap. Traps were eluted with 4 ml of dichloromethane (CH_2Cl_2), gently dried, reconstituted in 100 μl CH_2Cl_2, and transferred to a 150-μl glass insert of a 1.5-ml crimp-top glass vial. Samples were analyzed by GC-MS for MeJA content under the following conditions: fused silica-column (30 m $\times$ 0.25 mm) with a 0.25-μm DB-5 stationary phase held at 60°C for 4 min after injection (250°C), increased at 10°C/min to 200°C, followed by 20°C/min ramp to 300°C

for 7 min with He carrier gas maintained at 1 ml/min. Eluting compounds were detected by a Varian Saturn 2000 MS-MS ion trap (150°C) in electron-impact-ionization mode scanning masses 223-227. The trans and cis epimers eluted at 17.45 and 17.55 min, respectively (Preston et al., 2001). Since the flow rates were equal for all trappings, the results are presented as ng/hr.

While *A. tridentata* has several subspecies, all *A. tridentata* plants used in the experiments were *A. tridentata* ssp. *tridentata*. All collections were from naturally occurring sagebrush populations located in the Great Basin Desert of southwestern Utah [locations: township and range coordinates (section): kinetic T41S R18W (11); local vs. systemic T43S R18W (36); distance T43S R17W (6)]. Within each experiment, care was taken to choose plants of equal size and vigor.

The kinetic of the MeJA release was characterized by trapping the volatiles released by sagebrush in 2-hr intervals after a single damage event. Volatiles from eight sagebrush plants were first trapped from 8:30–10:30 A.M. to determine constitutive MeJA release. Each plant was then damaged by manually clipping the leaves contained within the enclosures with scissors (approximately 1–3 g of leaf material, 20% of the amount of total sagebrush leaf tissue enclosed was removed). Trappings were performed from 0–2, 2–4, 4–6, and 24–26 hr after damage.

To determine whether the MeJA release occurred from damaged leaves or systemically from undamaged leaves on damaged plants, four sagebrush plants located directly adjacent to each other and along a roadside were selected. Each plant was approximately 40–70 cm tall and 40–50 cm wide, and two to three other sagebrush plants on either side bordered the four plants. Two trapping containers were placed on opposite sides of each sagebrush plant to sample the headspace of the damaged portion separately from the undamaged portion of the plant. Branches were enclosed in trapping containers that remained on the plants for the entire experiment. One container from each plant was haphazardly assigned to either a damage treatment (local) or left undamaged (systemic), and volatile emissions were trapped for 4 hr first from the undamaged leaves of both branches before the damage treatment to determine if MeJA release was equivalent. Leaves in the local enclosure were then damaged by clipping with scissors and volatiles from all branches were trapped for 4 hr. Volatile emissions were again trapped 20 hr after damage for 4 hr to determine if there was a delayed increase in MeJA emissions from the systemic branches.

Naturally occurring tobacco plants neighboring damaged sagebrush suffered less herbivore damage as compared to tobacco neighboring undamaged sagebrush, but only when the plants were located within 10 cm of each other (Karban, 2001). To determine the distance over which the MeJA release is detectable, we trapped airborne MeJA emitting from single point source in the field (air temperature 36.6°C; soil temperature 50.5°C and a slight breeze). Four cotton wicks were impregnated with 500 μl MeJA each and positioned 20 cm above the ground. Volatile traps were positioned 10, 30, and 140 cm downwind from the cotton

swabs, and the volatilized MeJA was trapped for 5 hr. Of the MeJA placed onto the wick, approximately 19.8% was trapped at 10 cm, 7.3% at 30 cm, and 0.8% at 140 cm after 5 hr (Figure 2, inset). This exponential decay is consistent with the proposed model for dispersion of volatile compounds after release (Firn and Jones, 1995) and with the bioassay data; herbivore resistance decreases rapidly within a short distance from a damaged sagebrush canopy (Karban, 2001). Accordingly, we trapped volatiles at three distances from damaged sagebrush to determine the decay of the MeJA signal: directly within the sagebrush canopy, 20 cm and 40 cm from the sagebrush canopy. Traps were situated along a linear transect from the sagebrush out into a dirt road, such that no other sagebrush plants were located near any of the traps. Volatiles from eight undamaged sagebrush plants were collected for 8 hr, beginning at 11 A.M. The next day at the same time, the plants were damaged by clipping leaves along the entire canopy, removing approximately 5–8 g of leaf material, and volatiles were trapped for 8 hr. Plants were redamaged after 4 hr. For this experiment, the sagebrush branches were not placed within trapping containers, which would have disturbed the volatile dispersion.

To estimate the maximum MeJA volatile release from sagebrush plants, excised leaves (8.5–23 g) from 10 replicate 2-year old sagebrush plants were each placed into 4-l plastic chambers fitted with an activated charcoal trap containing 997 ng of internal standard positioned directly above each pile. Volatiles released by the material were trapped for 2 hr, and the release rate was calculated as ng/g/hr.

JA Analysis. Undamaged leaves from six sagebrush plants were collected separately in 2-ml microcentrifuge tubes, placed onto dry ice and stored at -20°C. The tissues were transported to the laboratory on dry ice and stored at -20°C until analyzed for JA content. JA was measured by GC-MS with [1,2-^{13}C] JA as an internal standard (Baldwin et al., 1997). Briefly, 436 ng of internal standard were added to approximately 0.15 g of leaf material and extracted first with 1.25 ml of extraction buffer, homogenized for 90 sec at 6.5 m/sec using the FastPrep® homogenizer (FP120; Q·Biogene, Heidelberg, Germany), centrifuged for 8 min at 13,000 rpm, and the supernatant transferred into a 4-ml glass vial. The leaf material was extracted again by adding 1.0 ml extraction buffer, homogenized as above, and centrifuged for 10 min at 13,000 rpm. JA contents of the pooled supernatants were analyzed by GC-MS after the clean-up procedures described in Schittko et al. (2000).

MeJA Analysis. To determine the size of the endogenous MeJA pools in undamaged sagebrush leaves, 3 replicates of approximately 0.2 mg sagebrush leaves were collected from a natural sagebrush population, and stored at -20°C, and extracted by soaking in 5 ml of CH_2Cl_2 for 5 min. Prior to adding CH_2Cl_2, each replicate received 1.42 μg of internal standard. The solvent was transferred to a clean 20-ml glass vial and gently dried. The extracted materials were reconstituted

in 100 μl CH_2Cl_2 and transferred into a 150-μl glass insert of a 1.5-ml crimp-top vial and analyzed by GC/MS as described earlier.

Plant Growth. Nicotiana attenuata [Torr. ex Wats. (synonymous with *Nicotiana torreyana* Nelson and Macbr.)] (Solanaceae) seeds, originally from bulk collections made from several plants growing in natural populations in Utah and inbred for three to seven generations in the glasshouse, germinated and grew for 14–17 days in a peat soil–perlite mixture soaked in 1:50 dilution of liquid smoke (House of Herbs, Passaic, NJ). Seedlings were transplanted and grown in either 250-ml or 2-l pots filled with a peat soil–perlite (approximately 3:1) mix of soil with 2 g (small pots) or 7 g (large pots) 14-14-14 N-P-K Osmocote slow-release fertilizer beads (Scotts Deutschland, Nordhorn, Germany) and 200 mg (small pots) and 1 g (large pots) Micromax micronutrient (Scotts Deutschland).

Herbivore Bioassay. M. sexta (Lepidoptera: Sphingidae) eggs were obtained from Carolina Biological Supply Company (Burlington, NC). Eggs were placed into rectangular polystyrene food containers (200-ml) with a clear lid (Neupack Verpackungen, Hamburg, Germany), lined with moist filter paper and maintained at 28°C, 65% relative humidity (RH), and a 16:8 hr light:dark photoperiod. Under these conditions, the eggs hatched 2–3 days after arrival. Larvae were used immediately after hatching and received no food prior to receiving the experimental leaf material. For the herbivore bioassays, one neonate larva was placed onto each treated leaf in separate bioassay containers.

Nicotine Analysis. Harvested tissues were flash frozen, lyophilized, ground to a fine powder, and 10-mg aliquots were used for nicotine extraction. Nicotine in the extracts was analyzed by high pressure liquid chromatography following methods described in Keinänen et al. (2001).

Protease Inhibitor Assay. Harvested plant tissue was flash frozen, lyophilized, ground to a fine powder, and 10–25-mg aliquots were extracted for PI activity. Each sample was analyzed for trypsin protease activity and protein content according to van Dam et al. (2001), except that the amount of extraction buffer was adjusted to maintain an equal ratio of plant material–extraction buffer.

MeJA Lanolin Treatments. Six days after transplanting into 250-ml pots, 10 plants were haphazardly assigned to each of three treatment groups: control, *trans*-MeJA [(original trans–cis ratio, 92:8; in lanolin, 100:0), Aldrich, Gillingham, Dorset, UK], and *cis*-MeJA [(original trans–cis ratio, 5:95; in lanolin, $63.34 \pm 0.99 : 36.66 \pm 0.99$), purified cis produced as described in Preston et al. (2001)]. Each MeJA epimer was applied to plants in concentrations of 0.5, 2.5, 10, and 25 μg per 20 μl of lanolin. Controls consisted of 20 μl lanolin. Ten plants were haphazardly assigned to each treatment, and the lanolin was applied in a thin strip across the leaf surface perpendicular to the mid-rib on the first fully expanded leaf. The amount of epimerization of trans and cis in the lanolin was estimated by measuring the MeJA in the headspace above each lanolin treatment. Lanolin,

containing either trans or cis, was aliquoted onto glass Petri dishes in 20 μl drops. Petri dishes were enclosed in transparent plastic 18.5-l chambers (Rubbermaid; Wooster, OH) each covered with a piece of UV transparent Plexiglas (UV-T). A trap was inserted alongside each Petri dish, and the volatile MeJA was trapped for 6 hr, and the volatiles were analyzed by GC/MS for the epimeric ratio. Due to the epimerization of the *trans*- and *cis*-MeJA in lanolin, the actual amounts of each epimer applied within each treatment were control, 0; 0.5 μg trans, 0.5 trans; 0.5 μg cis, 0.315 μg trans and 0.185 μg cis; 2.5 μg trans, 2.5 μg trans; 2.5 μg cis, 1.575 μg trans and 0.925 μg cis; 10 μg trans, 10 μg trans; 10 μg cis, 6.3 μg trans and 3.7 μg cis; 25 μg trans, 25 μg trans; and 25 μg cis, 15.75 μg trans, and 9.25 μg cis.

Two days after treatment applications, the treated leaf from each plant was harvested. Immediately after harvesting, leaves were divided in half parallel to the mid-rib. One leaf half was used in the herbivore bioassay and the other half was flash frozen in liquid N_2, lyophilized, ground to a fine powder, and analyzed for PI activity. PI activity is significantly induced 2 days after an initial MeJA treatment (van Dam et al., 2001). Four days after initial treatment, larvae were weighed, mortality was noted, and the leaf was replaced with the next youngest leaf from the same plant as the first leaf. The remaining rosette leaf of each plant was excised at the shoot–root interface and flash frozen in liquid N_2, lyophilized, finely ground, and analyzed for nicotine content. Nicotine concentrations are known to attain maximum values 4 days after MeJA treatment or wounding (Baldwin et al., 1998). Two days later, larvae were weighed, and mortality was recorded.

Aqueous MeJA Sprays. Three days after transplanting in 2-l soil pots, 7 plants were haphazardly assigned to each of the following treatments: control, trans low, trans high, cis low, cis high, trans–cis low, and trans–cis high. Controls received only sprays of water. Low MeJA treatments received 1 μg, while high MeJA treatments received 5 μg. The *trans*-MeJA treatments were produced by weighing the MeJA into a glass vial and adding sufficient water to produce the desired concentrations. The *cis*-MeJA is stored as a stock solution, with *cis* diluted in N-hexane. Dilutions were produced by measuring out the necessary volume of stock solution, allowing the solvent to evaporate completely, and adding the appropriate volume of water. Since MeJA is not soluble in water, all solutions were vigorously shaken before treating each plant. Treatments were delivered in five sprays of a glass perfume spray bottle, totaling 0.75 ml. To determine the amount of epimerization that occurs in the cis treatment during this procedure, 10 ml of the cis solutions were sprayed into glass scintillation vials and extracted twice with 10 ml CH_2Cl_2. The pooled solvent layer was gently dried, and the MeJA was reconstituted in 100 μl of CH_2Cl_2 and analyzed by GC-MS. After spraying, the trans–cis ratio in the trans treatments were 97.62 $\pm$ 0.5: 2.38 $\pm$ 0.5 and in the cis treatments, 36.9 $\pm$ 2.5: 63.1 $\pm$ 2.5. Treatments were applied every day for 7 days. In the last two treatments, plants received sprays of *trans*-MeJA each day for 5 days followed by

2 days of *cis*-MeJA. Over the 7 days of the experiment, the cumulative amount of MeJA, considering trans–cis epimeric ratios of 98:2 in the trans treatments and 37:63 in the cis treatments, received by each treatment was control, 0; trans low, 6.86 μg trans and 0.14 μg cis; cis low, 2.59 μg trans and 4.41 μg cis; trans–cis low, 5.64 μg trans and 1.36 μg cis; trans high, 34.3 μg trans and 0.7 μg cis; cis high, 12.95 μg trans and 22.05 μg cis; trans–cis high, 28.2 μg trans and 6.8 μg cis.

Seven days after the first treatment application, the first fully expanded leaf was excised at the petiole from each rosette, a technique that elicits only a minimal nicotine response (Baldwin et al., 1998). The leaf was bisected along the mid-rib. One half of each leaf was used in an herbivore bioassay. The other half was divided into two, perpendicular to the cut edge. The top half was analyzed for PI content, and the bottom half was analyzed for nicotine content. After 2 days, the next youngest leaf was similarly harvested. Larvae from the the first treatment leaf were weighed, mortality was noted, and then placed onto one-half of the leaf from the next harvest. Again, the top section of half of the leaf was analyzed for PIs and the bottom half for nicotine. Two days later, larvae were weighed and further mortality was noted. During the experiment, one plant in each control, cis low, and trans high group became diseased and were excluded from the analyses.

Statistical Analysis. One-way and two-way ANOVAs were used to analyze main effects and Fisher's PLSD post hoc tests were used to detect significant differences between groups when the original ANOVA was significant. Analyses were performed with the STATVIEW 5.0 statistical package (SAS Institute, Gary, NC).

RESULTS

JA and MeJA Leaf Concentrations. The concentrations of JA and MeJA are 3 and 4 orders of magnitude greater in excised leaves than the amount of MeJA emitted from undamaged sagebrush plants (Table 1). Sagebrush has

TABLE 1. MEAN ($\pm$1 SEM) AMOUNTS OF JA AND MEJA MEASURED WITHIN AND RELEASED FROM *Artemisia tridentata* SSP. *tridentata* LEAVES. CONSTITUTIVE AND DAMAGE-INDUCED MEJA RELEASE RATES FROM INTACT FIELD GROWN PLANTS ARE FROM KARBAN ET AL. (2000). N.D., NOT DETERMINED

Collection	*trans*	*cis*
Endogenous JA pools (μg g^{-1})	9.95 $\pm$ 1.40	n.d.
Endogenous MeJA pools (μg g^{-1})	19.08 $\pm$ 0.03	76.96 $\pm$ 0.77
Maximum release rates from detached leaves (ng g^{-1} h^{-1})	154.91 $\pm$ 28.94	975.01 $\pm$ 376.69
Constitutive release rates from undamaged plants (ng g^{-1} h^{-1})	21.9 $\pm$ 8.4	3.65 $\pm$ 0.73
Damage-induced release rates (ng g^{-1} h^{-1})	35.35 $\pm$ 9.79	34.43 $\pm$ 11.26

extraordinarily large endogenous JA pools (9.95 $\pm$ 1.40 μg/g) that are nearly 1000$\times$'s larger than that of *N. attenuata* (28.88 $\pm$ 5.17 ng/g; Schittko et al., 2000) and apparently has the ability to methylate and store large quantities, nearly 100 μg/g leaf material. Additionally, each hour 1.17% of this pool can be volatilized. Clearly, sagebrush is capable of releasing quantities of MeJA upon damage that would be physiologically active for *N. attenuata* plants growing nearby.

Characterizing MeJA Emissions

Kinetic of MeJA Emission After Damage. The total amount of MeJA released between the different trapping intervals was significantly different (Figure 1A; one-way ANOVA $F = 4.232$, $df = 4, 32$, $P = 0.007$). When compared to MeJA released by undamaged sagebrush, damage increased both the amounts of *trans*- and *cis*-MeJA released by sagebrush at 0–2 hr (P's = 0.003 and 0.005, respectively) and 2–4 hr (P's = 0.014 and 0.018, respectively) immediately after damage. After 4 hr, the damaged sagebrush's release of MeJA, either total, trans or cis, was no longer significantly different from undamaged levels (P's $\geq$ 0.08). Furthermore, there does not appear to be a delayed MeJA release, with the amount of MeJA detected 24–26 hr after damage being no different from that released from undamaged tissues (P's = 0.83 and 0.99 for trans and cis, respectively). We conclude that the maximum release of MeJA occurs within the first 4 hr following damage.

Local And Systemic Releases of MeJA After Damage. There was a difference in the quantities of MeJA released by the local and systemically located leaves after damage (Figure 1B; $F = 3.252$, $df = 5, 17$, $P = 0.031$). Damage caused an immediate increase in the total amount of MeJA released ($P = 0.010$), as well as in the trans or cis individually ($P = 0.012$ and 0.010, respectively) in the local treatment. The MeJA emissions from the systemically located leaves, either as total, trans or cis, remained similar to levels of MeJA released when the sagebrush was undamaged at both trapping intervals following damage (P's $\geq$ 0.65). At 20–24 hr after damage, MeJA emissions by local leaves were not significantly different than the MeJA levels of the same leaves before damage ($P = 0.84$). The increase in MeJA release after damage is clearly localized to the damaged leaves.

Distance from Sagebrush over Which MeJA is Detectable. A two-way ANOVA revealed a difference in the amounts of MeJA emitted by the undamaged and damaged sagebrush plants (Figure 1C; $F = 4.260$, $df = 1, 31$, $P = 0.048$), but no significant difference in the amount of MeJA trapped from the different distances to the sagebrush plants ($F = 0.444$, $df = 2, 31$, $P = 0.65$) or interaction between damage and distance ($F = 1.054$, $df = 2, 31$, $P = 0.75$). Moreover, the amount of trans and cis was not significantly different at each distance from either the undamaged sagebrush ($F = 0.542$, $df = 2, 12$, $P = 0.59$ and $F = 2.021$, $df = 2, 12$,

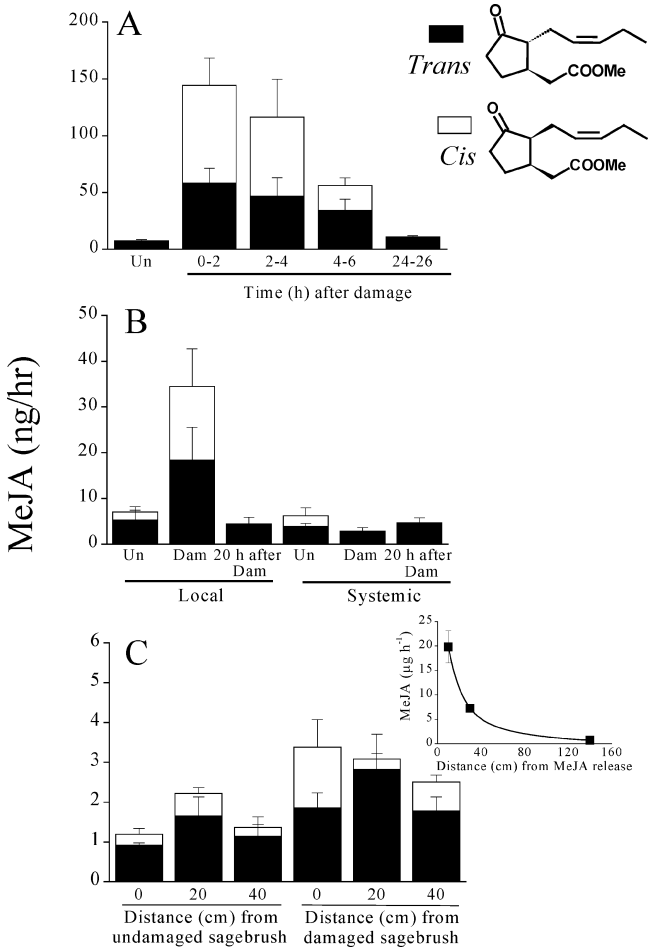

FIG. 1. Mean (+1 SEM) *trans* (black bar) or *cis* (white bar) MeJA trapped: A: from sagebrush branches before damage (Un) or 0–2, 2–4, 4–6, or 24–26 hr after a single mechanical damage; B: from branches that had either directly been damaged (local) or branches that were located on the same plant but were not themselves damaged (systemic); and C: at different distances from (0, 20, and 40 cm from the sagebrush canopy) to undamaged or damaged sagebrush plants. Inset: Amount of MeJA (mean +1 SEM, $N = 4$) trapped at three distances (10, 30, and 140 cm) downwind of a cotton wick impregnated with 500 μl MeJA. Branches in A and B were enclosed in 3.75-l plastic containers with air inlets at the bottom and outlets at the top. Sagebrush plants in C were not enclosed and located along a roadside such that there were no additional sagebrush plants were within 2 m of the traps. Volatile emissions were collected for 2 hr in A, 4 hr in C, 6 hr in C, and 5 hr in C (inset).

$P = 0.18$, respectively) or damaged sagebrush ($F = 0.995$, $df = 2, 19$, $P = 0.39$ and $F = 2.117$, $df = 2, 19$, $P = 0.15$, respectively). The amount of MeJA from undamaged and damaged sagebrush did not decline over a distance of at least 40 cm. While the amounts of MeJA trapped by our collection were low compared with the other trapping data (likely due to the lack of volatile collection chambers), we were able to detect MeJA at all distances. Two explanations may account for this. First, rather than assuming a diffusion-based exponential decay (i.e., Figure 1C inset), the volatile plume released by the sagebrush may remain intact as it is transported from the canopy. Second, the canopy is not a single point-source of volatile release, but rather a large surface of volatile release and the traps 20 and 40 cm from the canopy were sampling this continually produced cloud of MeJA.

Activity of cis- *and* trans-*MeJA in Lanolin Applications*

PI-Inducing Activity. Concentrations of PIs were different among the MeJA treatments (Figure 2A; $F = 5.302$, $df = 8, 61$, $P < 0.001$). The *trans*-MeJA treatment increased PI concentration at either 10 μg ($P = 0.030$) or 25 μg ($P = 0.003$) doses. However, the PI concentrations of cis-treated plants were above those of control plants at 2.5 μg ($P = 0.042$), and 25 μg ($P < 0.001$), but only marginally significantly at 10 μg ($P = 0.054$). cis was only more active than trans at the 25 μg level in increasing PI levels ($P = 0.013$).

Nicotine-Inducing Activity. As with PI concentrations, nicotine concentrations varied among the treatments (Figure 2B; $F = 6.462$, $df = 8, 80$, $P < 0.001$). Treatment with cis only marginally increased nicotine concentrations above those in control treatments at 2.5 μg ($P = 0.068$), 10 μg ($P = 0.068$), and 25 μg ($P = 0.074$). In contrast, trans treatments significantly increased nicotine concentration at 10 μg ($P = 0.001$) and 25 μg ($P < 0.001$) and trans was more active than cis ($P = 0.003$) at the highest application amount (25 μg). In summary, while cis was more active at the highest concentration in eliciting PIs, trans was more active in eliciting nicotine production than cis.

Hornworm Performance. Larvae fed leaves treated with the different epimers had equivalent mortality rates (Figure 2C), but their masses differed after they had fed on leaves from the different treatments for 2 and 4 days (Figure 2D; $F = 4.703$ and 2.705, $df = 8, 56$, $P < 0.001$ and $P = 0.014$, respectively). After 2 days, larvae consuming leaves treated with either 2.5 and 25 μg trans or 2.5, 10, and 25 μg cis were smaller than those feeding on control leaves (P's $\leq$ 0.030). The application of 0.5 μg of cis did not affect larval growth ($P = 0.083$). Only at the 10 μg level did larval masses differ significantly between trans and cis treatments ($P = 0.005$). After 2 days of consuming a systemically located leaf, larvae consuming leaves from plants treated with 25 μg trans or cis weighed less than those on leaves from control plants (P's $= 0.014$ and 0.051,

respectively). Larvae fed leaves from the 10 μg cis treatment grew less than those consuming leaves from the 10 μg trans treatment ($P = 0.049$). While cis may be slightly more active than trans, the effects are not consistent at all treatment levels.

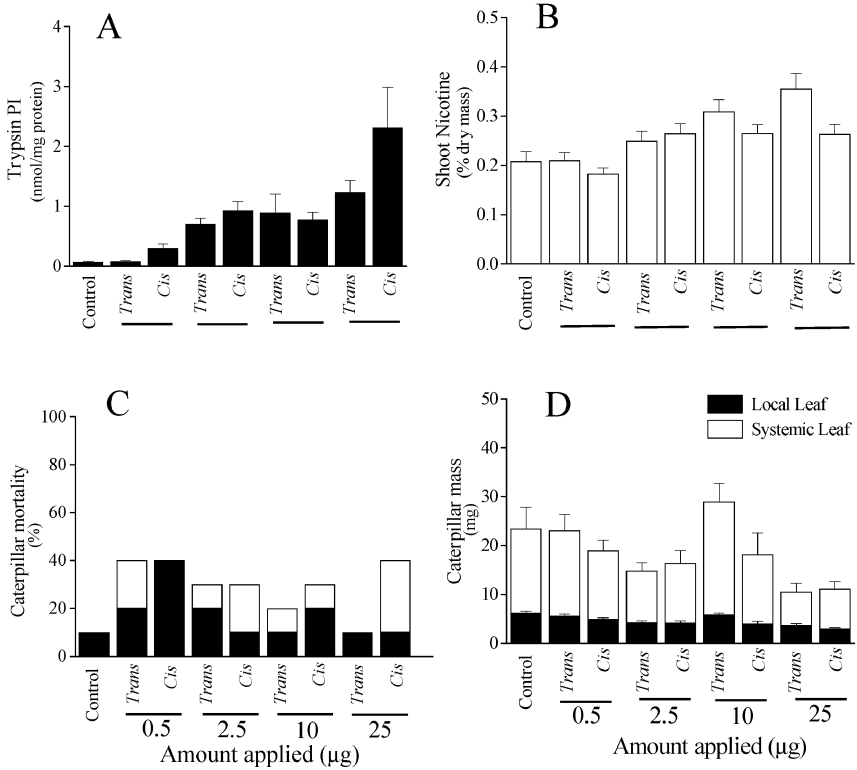

FIG. 2. A: Trypsin PI levels (mean +1 SEM) in leaves of *N. attenuata* plants treated 2 days earlier with lanolin applications of either *trans*- or *cis*-MeJA at 0.5, 2.5, 10, or 25 μg per plant. See Methods section for an estimate of applied values of each MeJA epimer that includes epimerization of *trans*- to *cis*-MeJA during application. Control plants received only treatments of lanolin. B: Shoot nicotine levels of plants 4 days after treatment applications. C: Mortality of *M. sexta* larvae on the treated (black bar) and systemic leaf (white bar), harvested 2 and 4 days, respectively, after treatments were applied. Percentage mortality is expressed as the cumulative number of larvae in each treatment that died of the initial 10 larvae. D: Mass of *M. sexta* larvae 2 days after feeding on the treated leaf (black bar) and 2 days after feeding on the systemic leaf (white bar).

Activity of cis- *and* trans-*MeJA in Aqueous Spray Applications*

PI-Inducing Activity. PI concentrations of the first leaf harvested were different between the treatments (Figure 3A; $F = 4.850$, $df = 6, 37$, $P = 0.001$), with significant differences between the control and the trans high ($P < 0.001$), cis high ($P = 0.001$), and trans–cis low ($P = 0.005$) treatments. No differences between the trans and cis, at either the low dose ($P = 0.87$) or high dose ($P = 0.51$) were found.

In contrast, PI levels in the second leaf harvested were not different among the treatments (Figure 3B; $F = 1.785$, $df = 6, 37$, $P = 0.13$). The results do not support the differences between epimers observed in the lanolin treatment or the hypothesis that the trans to cis switch represents a uniquely active signal.

Nicotine-Inducing Activity. Nicotine concentrations in the first leaf harvested were different between the treatments (Figure 3C; $F = 3.026$, $df = 6, 39$, $P = 0.016$). The trans–cis low treatment increased nicotine concentrations above that found in the control treatment ($P = 0.005$), while those in the trans high and trans–cis high treatments were only marginally greater than that of controls ($P = 0.053$ and 0.062, respectively). At low and high doses, trans and cis treatments were not significantly different. However, nicotine levels in the trans–cis low treated leaves were greater than those in the trans low ($P = 0.02$) and cis low ($P = 0.001$) treatments.

Nicotine concentrations in the second leaf harvested were different among the treatments (Figure 3D; $F = 2.893$, $df = 6, 38$, $P = 0.020$). Only the trans–cis high was higher than controls ($P = 0.003$). The nicotine concentration in the trans–cis high treatment was also greater than that in trans high ($P = 0.030$) and cis high ($P = 0.003$). In summary, the trans to cis switch appears to elicit nicotine production more effectively than either epimer alone.

Hornworm Performance. Overall, larval mortality remained lower and larval weight gain larger than in the lanolin-treatment experiment (Figure 3E). Larval masses among the different treatments were not significantly different after 2 days of consuming the first leaf (Figure 3F; $F = 0.978$, $df = 6, 36$, $P = 0.454$). However, larval masses among all of the treatments were significantly different after 4 days of consuming treated leaf material, 2 days on the first harvested leaf and 2 days on the second harvested leaf (Figure 3F; $F = 2.486$, $df = 6, 36$, $P = 0.04$). Larvae feeding on leaf material from the trans high, cis high, and trans–cis high treatments weighed less than those feeding on leaves from control treatments (P's $= 0.005$, 0.048, and 0.026, respectively). There were no differences between the trans, cis, or trans–cis treatments at either dosage level (P's ≥ 0.34). Differences in larval growth between the different MeJA treatments was not observed after larvae fed on the second leaf for 2 days.

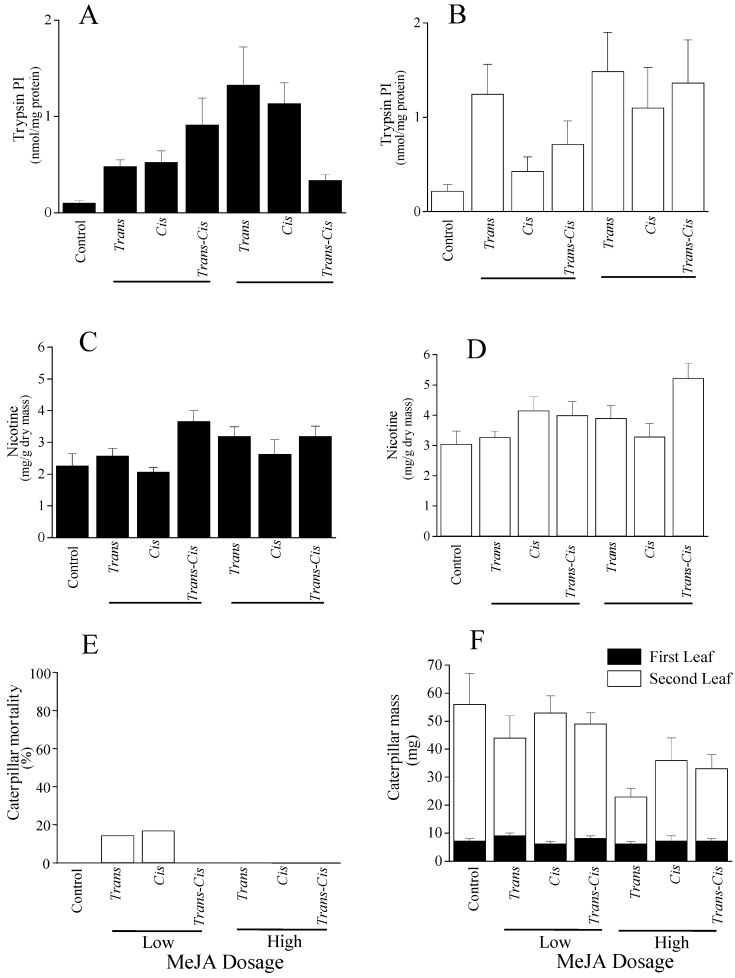

FIG. 3. Trypsin PI (A, B) and nicotine (C, D) levels (mean +1 SEM) in the first fully expanded leaf harvested from each plant 7 days after initiation of treatments (2 days after the first cis application in trans–cis treatment), and in the leaf at the next youngest node 2 days after the first harvest (9 days after initiation of treatments), respectively. Plants were treated daily with either 1 μg (low) or 5 μg (high) of their respective epimers. See Methods section for an estimate of applied values of each MeJA epimer that includes epimerization during application. E: Percentage mortality of *M. sexta* larvae on the first (black bar) and second leaf (white bar), harvested 7 and 9 days after treatments were initiated, respectively. Mortality is expressed as the cumulative number of larvae in each treatment that died of the initial 7. F: Mass of *M. sexta* larvae 2 days after feeding on the first leaf (black bar) and 2 days after feeding on the second leaf (white bar).

In summary, our results are not consistent with the hypothesis that cis is biologically more active than trans in eliciting resistance against this specialist herbivore.

DISCUSSION

Studies of plant-plant communication generally fail to treat plants with relevant quantities of the chemical of interest in a manner that mimics the natural release. Experimental treatments are often several orders of magnitude higher than natural exposures and plant responses are tested in small, enclosed containers. For example, Birkett et al. (2000) placed 2.5 mg *cis*-jasmone (their putative interplant signal) on a filter paper in a closed container with 9 plants for 24 hr, during which time the compound completely volatilized. No values are reported for the natural release rate of *cis*-jasmone from *Ribes nigrum* (the focus of the study), but the experimental release rate (104.17 μg/hr, assuming an equal emission rate over the exposure interval) is 4 orders of magnitude greater than that measured from six varieties of cotton (2.1 $\pm$ 4.0 to 19.3 $\pm$ 13.7 ng/hr; Loughrin et al., 1995). Similarly, Arimura et al. (2000a) exposed excised lima bean leaves to 10 μg of their test compounds for 3 or 24 hr in a 7-l sealed chamber. When infested with spider mites, lima bean plants release approximately 99.2, 122, and 164.4 ng/hr/plant of (*E*)-β-ocimene, (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT), and (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), respectively (Dicke et al., 1999). It is not clear how quickly the volatiles were emitted from the cotton wool. In addition to using high exposures, both studies contained plants (or excised leaves) in small air-tight containers under illuminated conditions in which plants would likely draw down the CO_2 concentration below their CO_2 compensation point, increase stomatal openings, and further exaggerate the exposure of the "receiver" leaves to volatile signals. In summary, the experimental procedures used in laboratory tests of interplant signaling have tended to favor the accumulation of volatile compounds in the headspace of receiver plants, rather than having them disperse, as would occur in natural environments.

Of plants examined for MeJA production, *Artemisia* and *Jasminum* are unique in that they release large quantities of MeJA and, unlike most plants, wounding is not required for production (Hildebrand et al., 2000). The pools of JA are substantial (Table 1), among the highest concentration reported for any plant (Farmer, 1994; Mueller, 1997) and are nearly three orders of magnitude greater than that found in undamaged *N. attenuata*. Additionally, even larger pools of MeJA exist; approximately 96 μg/g with a trans–cis ratio of 20:80 (Table 1). It is unknown how MeJA is formed within the cell, but work in snapdragon has identified a methyl ester-forming enzyme, *S*-adenosyl-L-methionine/benzoic acid carboxyl methyl transferase, responsible for the formation of the volatile methyl

ester, methyl benzoate, from benzoic acid (Dudareva et al., 2000). Presumably, a similar enzyme methylates the large pools of free JA in the plant prior to its release. The clean-up procedure for the JA determinations epimerizes the endogenous JA pool (Baldwin et al., 1997), so it is not possible to determine whether the endogenous JA pool is predominantly cis or trans. However, the majority of the evidence points to *cis*-JA as the first biosynthetic product in plants (Mueller and Brodschelm, 1994). This is further supported by the high proportion of MeJA existing as cis in undamaged sagebrush leaves (Table 1). Large pools of JA are likely to progress toward their thermodynamic equilibrium of 92:8, possibly as a consequence of epimerization promoted by unspecific or specific protein binding in the plant cell (Mueller and Brodschelm, 1994). It is likely that these large pools of JA and MeJA provide an unlimited source for MeJA emission from sagebrush.

To be an effective airborne signal, the active component must contain accurate information and remain at physiologically active levels over a biologically-relevant distance. Much like the release of the "green leaf" volatiles [also proposed as possible airborne signals (Bates and Rothstein, 1998; Engelberth et al., 2004)], the increased emission of MeJA and, in particular, *cis*-MeJA is tightly associated with damage, occurring immediately after damage (Figure 1A) and only from damaged tissues (Figure 1B). Moreover, the amount of MeJA at the site of release (sagebrush) is not significantly reduced for at least 40 cm (Figure 1C), suggesting that the damage-induced enrichment of MeJA in the immediate headspace of an attacked sagebrush is not likely to be diluted at biologically relevant distances. This contrasts with the expected exponential decay in airborne concentrations with distance as volatiles diffuse from a point source (Figure 1C inset). Sagebrush plants are, however, not point sources and their canopy provides a structure that may affect local air currents. In summary, our data support the hypothesis that *A. tridentata* is capable of releasing substantial quantities of volatile *cis*-MeJA and the release of *cis*-MeJA fits some of the expectations for an effective airborne signal. Whether or not receiver plants respond is the second question we addressed.

We developed two procedures that delivered *cis*- and *trans*-MeJA in realistic quantities directly to plants: (1) in a lanolin paste, which delivered a whole-plant dose to a defined leaf area in a single exposure and, (2) in an aqueous spray, which delivered smaller amounts to the entire canopy in a series of applications to simulate an extended exposure. The two treatment methods may also deliver the two isomers with different probabilities of epimerization before they enter the plant. Lanolin applications may provide a lipophilic route into the plant that avoids the enolization required for epimerization (Mueller and Brodschlem, 1994). Enolization occurs during protonation of the C-6 keto group, which might occur with a greater frequency in the aqueous spray application. Headspace trapping (trans–cis ratio of 63:37) of the lanolin containing the cis treatment with an original trans–cis

ratio of 5:95, provided evidence for significant epimerization (61% of cis) either in the lanolin or during volatilization from the lanolin into the air. A similar analysis of the aqueous sprays revealed less epimerization (34% of cis). However, it is not clear which application procedure simulates the epimerization that occurs when plants are exposed to airborne MeJA. Lanolin applications provided some support that *cis*-MeJA is biologically more active than trans because concentrations of PIs and larval mortality were greater and larval mass lower in plants treated with cis (Figure 2A and C). However, aqueous sprays did not support these conclusions, but rather demonstrated that trans was approximately as active as cis in eliciting PI accumulation (Figure 3A and B). Previous studies testing the different epimers in various bioassays suggest that each may activate different jasmonate receptors and elicit different responses (Koda et al., 1992; Weiler et al., 1993). For example, growth inhibition and production of the secondary metabolites, paclitaxel and baccatin III, in *Taxus* cells is differently regulated by the different MeJA epimers (Yukimune et al., 2000). We conclude that the different MeJA epimers may trigger different responses in plants, but their overall activity does not differ dramatically. Moreover, prior exposure to trans, as would occur when plants grow next to undamaged sagebrush, does not dramatically sensitize plants to a short-term exposure to cis, as would occur when sagebrush is damaged (Figure 3A–F). In summary, the amount of MeJA applied to plants, rather than the particular epimer applied, was the most important determinant of plant responses in these laboratory experiments. Whether the quantities used realistically represent natural exposures deserves further discussion.

A damaged sagebrush plant releases approximately 40–80 ng/g/hr MeJA (Karban et al., 2000) from its damaged tissues. It is reasonable to assume that on average, an attacked sagebrush plant would have 10 g of damaged leaves in its canopy releasing at this rate. Such a damaged sagebrush plant would release 400–800 ng/hr MeJA and if a nearby tobacco plant received this entire dose for as long as the herbivores attacked the sagebrush, a plant would receive an amount comparable to that received in the aqueous spray treatments, which delivered 1 or 5 μg MeJA each day. At these doses, both PI and nicotine concentrations were elevated above controls, and growth of *M. sexta* larvae was negatively influenced. While both of these traits are known to be correlated with plant fitness and resistance in natural populations of *N. attenuata* (Baldwin, 1998; Glawe et al., 2003), it is unclear whether the responses are sufficiently strong to account for the decreased herbivory observed in the Karban et al. (2000) study. In a field study (Baldwin, 1998), 500 μg applications of MeJA (90.1: 8.3; trans–cis) to the rhizosphere of plants were required to induce nicotine concentrations comparable to that elicited by foliar wounding. In this study the MeJA was delivered in 10 ml of water to the soil surrounding the plant's roots, and it is not clear how much actually came in contact with plant roots or was volatilized and assimilated by the shoot. It is clear, however, that the entire quantity of MeJA released from a plant will not be directly

deposited onto a single neighboring tobacco plant. Without trapping containers to contain the headspace from a damaged sagebrush, the quantities of MeJA trapped were about 10-fold less than those trapped from undamaged sagebrush and 10- to 50-fold less than those from the damaged sagebrush (Figure 1C). At these concentrations (1–3 ng/hr) we have no evidence that MeJA elicits resistance or resistance-related traits. A field experiment was set up to examine responses of natural populations of tobacco to the two epimers, but unfortunately, an outbreak of *M. quinquemaculata* and *M. sexta* prevented us from detecting differences in MeJA-treated plants compared to controls. All plants in the experiment were completely consumed shortly after treatment applications (Preston et al., 2001).

Additional field studies are needed to determine whether sagebrush's wound-induced MeJA release effects resistance in nearby growing tobacco. Moreover, it will be important to quantify MeJA release in response to herbivore attack and not simply to mechanical wounding, which was used in the Karban et al. (2000) study. Other wound-induced volatile releases are amplified and quantitatively altered when herbivores cause the wounding or herbivore-specific salivary factors are added to wounds (De Moraes et al., 1998; Halitschke et al., 2000, 2001). However, since wounding does not increase endogenous MeJA or JA levels above the already high constitutive titers found in sagebrush (Hildebrand et al., 2000), it would be surprising if herbivory dramatically altered release rates.

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IDENTIFICATION OF VOLATILES THAT ARE USED IN DISCRIMINATION BETWEEN PLANTS INFESTED WITH PREY OR NONPREY HERBIVORES BY A PREDATORY MITE

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Abstract—Carnivorous arthropods can use herbivore-induced plant volatiles to locate their herbivorous prey. In the field, carnivores are confronted with information from plants infested with herbivores that may differ in their suitability as prey. Discrimination by the predatory mite *Phytoseiulus persimilis* between volatiles from lima bean plants infested with the prey herbivore *Tetranychus urticae*, or plants infested with the nonprey caterpillar *Spodoptera exigua*, depends on spider mite density. In this article, we analyzed the chemical composition of the volatile blends from *T. urticae*-infested lima bean plants at different densities of spider mites, and from *S. exigua*-infested plants. Based on the behavioral preferences of *P. persimilis* and the volatile profiles, we selected compounds that potentially enable the mite to discriminate between *T. urticae*-induced and *S. exigua*-induced volatiles. Subsequently, we demonstrated in Y-tube olfactometer assays that the relatively large amounts of methyl salicylate and (3*E*, 7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene emitted by *T. urticae*-infested bean plants compared to *S. exigua*-infested plants enable the predators to discriminate. Our data show that specific compounds from complex herbivore-induced volatile blends can play an important role in the selective foraging behavior of natural enemies of herbivorous arthropods.

Key Words—Tritrophic interactions, food webs, herbivore-induced plant volatiles, terpenes, methyl salicylate, indirect plant defense, spider mites, *Phytoseiulus persimilis*.

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INTRODUCTION

Carnivores that search actively for food need information on the whereabouts of their prey. The information emitted by the prey is often minimal because of selection to reduce the risk of predation. Carnivores can solve this problem by using information from the prey's direct environment instead of from the prey itself. An intriguing and well-established example of this phenomenon is the use of information from plants by carnivorous arthropods. Upon attack by herbivorous insects or mites, plants emit a blend of volatiles that can attract a range of different carnivore species, including parasitoid wasps and predatory mites and bugs (e.g., Dicke et al., 1990; Turlings et al., 1990; Vet and Dicke, 1992; Paré and Tumlinson, 1997; Scutareanu et al., 1997; Drukker et al., 2000).

Information emitted by the food plants of prey herbivores is relatively easy to detect because plants have a large biomass compared to herbivores, and plants can benefit from the emission of volatiles by attracting carnivorous arthropods (Dicke and Sabelis, 1989; Vet and Dicke, 1992; Van Loon et al., 2000; Fritzsche Hoballah and Turlings, 2001). However, this indirect information is often more variable than information from the prey itself. Variation in the composition of herbivore-induced plant volatile blends can be related to plant species and cultivar, herbivore species, multiple infestation by another herbivore species or pathogen, and abiotic factors (e.g., Takabayashi et al., 1994; DeMoraes et al., 1998; Gouinguené and Turlings, 2002; Cardoza et al., 2002; Rodriguez-Saona et al., 2003; Schmelz et al., 2003). To foraging carnivores, it is especially important to attend to the differences or variation in volatile blends that are associated with herbivore species because the herbivores may differ in their suitability as prey. Field and laboratory studies have shown that carnivores can use herbivore-induced plant volatiles to discriminate between the volatiles from plants infested with prey and plants infested with non-prey herbivore species (e.g., Du et al., 1996; DeMoraes et al., 1998). However, no studies have yet conclusively shown which differences in volatile blend composition enable carnivores to make this choice (e.g., Chadwick and Goode, 1999; Dicke and Van Loon, 2000).

Here, we aim to identify which differences between the volatile blends of lima bean plants infested with the two-spotted spider mite (*Tetranychus urticae*) or the beet armyworm (*Spodoptera exigua*) may enable the predatory mite *Phytoseiulus persimilis*—a specialized natural enemy of spider mites—to discriminate between these blends. We recently showed that discrimination by *P. persimilis* between these blends depends on mite density: the predators prefer the volatiles induced by their prey *vs*. those induced by the nonprey herbivore when spider mite density is high (i.e., 40 per leaf), but not when mite density is low (i.e., 10 per leaf) (De Boer et al., 2004). In the present study, we analyzed the chemical composition of the volatile blends emitted by lima bean plants upon infestation with the different densities of *T. urticae*, and one density of *S. exigua*. On the basis of differences in

the chemical composition of the blends, we selected compounds that potentially enable the predators to discriminate. Subsequently, we evaluated the role of these compounds in Y-tube olfactometer experiments.

METHODS AND MATERIALS

Plants and Herbivores. Lima bean plants (*Phaseolus lunatus* L. cv. Sieva) were grown in the greenhouse at 20–30°C, 50-70% R.H., and 16 hr light. They were used in experiments when their primary leaves had expanded, which was 10–15 days after sowing. A colony of the two-spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) was maintained on lima bean in the greenhouse under the same conditions. Eggs of *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) were obtained from the laboratory of Virology, Wageningen University, The Netherlands (Smits et al., 1986). Two to three egg batches were placed in a Petri dish with a lima bean leaf and kept in a climate cabinet at 23 ± 1°C and 50–70% R.H. Larvae were used to infest plants within 24 hr of hatching.

Predators. A stock colony of the predatory mite *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae) was kept on mite-infested lima bean leaves in a climate cabinet at 23 ± 1°C, 50–70% R.H., and continuous light. To obtain females of the same age, we transferred about five gravid females from the stock colony to a fresh *T. urticae*-infested lima bean leaf in each of a series of Petri dishes. The females were allowed to oviposit for 1 or 2 days and were then removed. Fresh mite-infested leaves were provided every 2 days to rear the offspring. Offspring females were used in experiments 9 days after initiation of the colonies, i.e., 1–2 days after their final molt. Prior to an experiment, females were kept individually in Eppendorf vials for 24 ± 2 hr, without food but with a small droplet of water. Petri dishes and Eppendorf vials were kept under the same conditions as the stock colony of predators.

Odor Sources. The two primary leaves of a lima bean plant were infested with caterpillars or spider mites 3 days before an experiment. Newly hatched caterpillars were placed in a clip cage that was attached to a leaf. Adult spider mite females from the greenhouse colony were transferred to the test plants using a fine paintbrush. An empty clip cage was attached to each mite-infested leaf as a control. Clip cages were supported with sticks to prevent the leaves from bending down and incurring damage by the weight of the cages. We moved clip cages to a new position on the same leaf daily, checked the number of caterpillars, and if necessary added a new caterpillar of the same age. Plants were incubated in a climate room at 24 ± 1°C, 50–70% R.H., and 16L:8D. Plants of different treatments were kept separate in plastic cages. The numbers of herbivores that we used are specified below for the volatile collections and the olfactometer experiments.

Volatile Collection and Analysis. We collected the volatiles of uninfested lima bean plants, *T. urticae*-infested plants (with 10 or 40 spider mites per leaf), and *S. exigua*-infested plants (two caterpillars per leaf). After 3 days of incubation, plants were cut and their stems were wrapped in wet cotton wool and aluminum foil. All clip cages, caterpillars and their products were removed. The plants were immediately subjected to headspace sampling. Five plants of one treatment were transferred to a 5 l glass vessel. A viton O-ring and a metal clamp were used to attach the glass lid airtight to the vessel. Purified air (filtered through silica, a molecular sieve [pore diameter 4 Å], activated charcoal, and 90 mg Tenax) was split into two air streams of about 100 ml/min and led into the vessels through Teflon tubing. In this way, volatiles from two treatments could be collected simultaneously in parallel. Volatiles from plants of the other two treatments were collected on the same day. The system was purged for 30 min before attaching a tube filled with 90 mg Tenax to the air outlet in the lid to trap the volatiles. Volatiles were collected for 25 min at 21 $\pm$ 1°C. We repeated the volatile sampling on four different days but, during the analysis, we lost one replicate of the "uninfested" and "10 spider mites" treatments each.

Volatiles were released from the Tenax traps with a thermodesorption coldtrap set-up (Chrompack, Middelburg, The Netherlands) by heating at 250°C for 10 min, with a He-flow of 12 ml/min. The desorbed compounds were collected in a cold trap at −90°C. Volatiles were injected in splitless mode into a DB5 column (60 m × 0.25 mm i.d., 0.25 μm film thickness) by heating of the cold trap to 220°C. After an initial column temperature of 40°C for 4 min, the temperature was raised to 280°C at 4°C/min. The column was directly coupled to the ion source of a Finnigan MAT 95 mass spectrometer, which was operating in the 70 eV EI ionization mode and scanning from mass 24–300 at 0.5 scans/sec. Compounds were identified by comparison of mass spectra with those in the NIST 98 library and in the Wageningen Mass Spectral Database of Natural Products, and by checking the retention indices. The peak areas were assessed for compounds that were present in at least N-1 replicates of at least one of the treatments.

Y-Tube Olfactometer Experiments. Responses of predatory mites to herbivore-induced volatiles were tested in a closed system Y-tube olfactometer (Takabayashi and Dicke, 1992). In short, two streams of purified air (filtered through activated charcoal) were each blown through a 2 l glass container and into the olfactometer arms at 4 l/min. The base of the olfactometer was connected to house vacuum at 8 l/min. We introduced an individual female predator at the base of the olfactometer by placing it on a metal wire running through the center of the tube. Each predator was observed for a maximum of 5 min, and a choice for one of the two odor sources was recorded when it reached the finish line halfway up one of the olfactometer arms. When the female did not make a choice within 5 min, a "no-choice" was recorded. Odor sources were interchanged after every 5–10 predatory mites to avoid any influence of unforeseen asymmetries in the setup.

The olfactometer and glass containers were cleaned after every experiment. Each experiment was repeated on 3–8 different days with new odor sources and new sets of predatory mites on each day. All olfactometer experiments were conducted at $21 \pm 1^{\circ}$C.

We selected the four largest peaks that were detected in the volatile blend of *T. urticae*-infested lima bean leaves to evaluate their contribution to the attractiveness of this blend: (3*E*)-4,8-dimethyl-1,3,7-nonatriene [(*E*)-DMNT], methyl salicylate [MeSA], (3*E*, 7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene [(*E*, *E*)-TMTT], and 2-butanone (see Results section). MeSA (Merck, 99% pure) and 2-butanone (Sigma, $\geq$99% pure) were obtained commercially; (*E*, *E*)-TMTT and (*E*)-DMNT were kindly provided by Dr W. Boland of the Max Planck Institute for Chemical Ecology, Jena, Germany. The purity of the latter compounds was measured with gas-chromatography and was at least 95%. To avoid the use of solvents and to ensure a stable release rate, we used microcapillaries to release the synthetic compounds into the olfactometer. To this end a 0.5 μl glass microcapillary (Drummond Scientific, USA) was filled with a pure compound and was placed in the last glass compartment of the olfactometer arm at an angle of $\sim 45^{\circ}$ relative to the direction of the air stream. The release rates of the synthetic compounds were estimated by measuring the length of the capillary over which the compound had evaporated during a certain time and calculating this to weight/hr evaporated. Release rates were 10 μg/hr for MeSA, 0.5 μg/hr for (*E*, *E*)-TMTT, 4 mg/hr for 2-butanone, and 11 μg/hr for (*E*)-DMNT. Because 2-butanone was highly volatile, the microcapillary had to be refilled after testing two to three predators; microcapillaries with the other compounds were not replaced for the duration of one experiment.

First, we tested the attractiveness of the four single compounds. A microcapillary with a synthetic volatile was presented in one arm of the olfactometer, while no odor was presented in the other arm of the olfactometer. Different compounds were tested on different days. The experiments were repeated on 5–8 different days with 8–14 predatory mites per day.

Second, we evaluated the contribution of the four compounds to the attractiveness of the volatile blend induced by the prey herbivore by offering predatory mites the choice between natural *T. urticae*-induced lima bean volatiles *vs*. one–three synthetic volatiles. Lima bean leaves were infested with 40 spider mites per leaf. Leaves were detached from the plant just before an experiment and were individually wrapped in wet cotton wool and aluminum foil. The alternative odor source consisted of (A) no odor, (B) MeSA, (C) (*E*, *E*)-TMTT, (D) 2-butanone, (E) (*E*)-DMNT, (F) MeSA + (*E*, *E*)-TMTT, (G) MeSA + (*E*, *E*)-TMTT + 2-butanone, (H) MeSA + (*E*, *E*)-TMTT + (*E*)-DMNT, (I) MeSA + (*E*, *E*)-TMTT + (*E*)-DMNT + the volatiles from four uninfested lima bean leaves. The control (A) was repeated on 18 different days with 8–10 predators per day. The other combinations were each repeated on 3–6 different days with 8–20 predators per day. Two–four

combinations of synthetic compounds were tested per day against the volatiles from the same set of *T. urticae*-infested leaves.

Finally, we investigated the role of three compounds in the discrimination by the predators between the volatiles from *T. urticae*-infested and *S. exigua*-infested lima bean. MeSA and (*E*, *E*)-TMTT were selected because they were emitted in a significantly larger amount by *T. urticae*-infested bean plants than by *S. exigua*-infested plants at the mite density that resulted in discrimination by *P. persimilis* between the two odor sources. 2-Butanone was included in this experiment as a control for a nonspecific effect of adding an extra compound to the volatile blend induced by *S. exigua*-induced. 2-Butanone was emitted in similar amounts by *T. urticae*-infested and *S. exigua*-infested plants, and it was not expected to play a role in discrimination by *P. persimilis*. Lima bean leaves were infested with 40 *T. urticae* or 2 *S. exigua* larvae per leaf. First, we determined the choice of about 10 predators between these two odor sources. Then, we placed a microcapillary with one of the synthetic volatiles in the olfactometer, downwind of the caterpillar-infested leaves. In this way, we tested the response of about 20 predators to the *T. urticae*-induced volatiles *vs.* the combination of *S. exigua*-induced volatiles plus the synthetic volatile. We subsequently removed the microcapillary with the synthetic volatile and replaced the olfactometer for a clean one before testing another set of about 10 predators for their choice between *T. urticae*-induced and *S. exigua*-induced volatiles. Different synthetic volatiles were tested on different days. We repeated this experiment on 4–8 different days for each of the three synthetic compounds.

Data Analysis. Differences in the emission of volatile compounds from lima bean plants infested with 40 *T. urticae* or with 2 *S. exigua* were analyzed with a Mann–Whitney *U* test.

Predatory mites that did not make a choice in the olfactometer (in total about 4% of all predators) were excluded from the statistical analysis. To investigate whether the choices of predators between the odor sources in the olfactometer differed from a 50:50 distribution, we used a two-sided binomial test on the pooled data per experiment. We used a generalized linear model (GLM) to comparatively analyze the effect of combining *S. exigua*-induced volatiles with a synthetic compound, testing the null-hypothesis that adding the extra compound does not change the behavior of predatory mites. We used a GLM with a binomial distribution and a logit-link function (GenStat 6.1, McCullagh and Nelder, 1989). The number of predatory mites choosing for the volatiles from *T. urticae*-infested bean per day was set as the response variate. The total number of predators that made a choice per day was taken as the binomial total. The predicting factors were replicate and odor source. We checked the validity of the models by comparing the deviance of the residual with the critical χ^2-value at $\alpha = 0.05$. The predicting factors and/or variates that we used to analyze each experiment are indicated in the figure legends.

RESULTS

Volatile Analyses. Plants that had been left uninfested or had been infested with 10 *T. urticae* per leaf emitted small amounts of (*Z*)-3-hexen-1-ol acetate, methyl salicylate (MeSA), (3*E*)-4,8-dimethyl-1,3,7-nonatriene [(*E*)-DMNT], and (3*E*, 7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene [(*E*, *E*)-TMTT] (Figure 1A and B). When 40 spider mites per leaf were used, a larger number of compounds was detected in addition to the compounds mentioned before: 2-butanone, (*E*)-β-ocimene, (*Z*)-DMNT, a TMTT-isomer, ethyl salicylate, and two unknown compounds. Moreover, the emission of MeSA, (*E*)-DMNT and (*E*, *E*)-TMTT increased about sixfold compared to the emission of bean plants infested with 10 spider mites per leaf (Figure 1C).

The major compound emitted by plants that had been infested with 2 *S. exigua* larvae per leaf was (*E*)-β-ocimene (Figure 1D). Large amounts of (*Z*)-3-hexen-1-ol acetate and (*E*)-DMNT were emitted as well. A range of compounds that were not detected or only in trace amounts upon mite-infestation, were emitted upon caterpillar-infestation: i.e., 2- and 3-methylbutanal-*O*-methyl oxime, (*Z*)-β-ocimene, β-caryophyllene, *p*-mentha-1,3,8-triene, and indole.

Compared to plants infested with 40 spider mites per leaf, significantly smaller amounts of MeSA, (*E*, *E*)-TMTT, and decanal were emitted by caterpillar-infested plants, whereas significantly larger amounts of (*E*)- and (*Z*)-β-ocimene were emitted by caterpillar-infested plants (Mann–Whitney *U* test, $P < 0.05$).

Olfactometer Experiments. First, we tested the attraction of the predators to the four largest peaks that were emitted by *T. urticae*-infested bean plants. Predators were strongly attracted to the single pure compounds MeSA (Figure 2, $P = 0.004$, binomial test) and 2-butanone ($P < 0.001$), while the attraction to (*E*, *E*)-TMTT bordered significance ($P = 0.07$), and *P. persimilis* was not attracted to (*E*)-DMNT ($P = 0.95$).

Second, we tested the attractiveness of the same four compounds *vs*. the natural blend of spider mite-induced volatiles. The choice of predatory mites between synthetic MeSA and the natural volatile blend from *T. urticae*-infested bean leaves was not significantly different from a 50:50 distribution (Figure 3B, $P = 0.20$, binomial test). (*E*,*E*)-TMTT, 2-butanone or (*E*)-DMNT did not have such an effect: the predators significantly preferred the volatiles from *T. urticae*-infested leaves to either of these pure synthetic compounds (Figure 3C–E, $P < 0.001$, binomial test). MeSA was part of all the mixtures of synthetic compounds tested and the predators did not discriminate between these blends of synthetic volatiles and the volatiles from *T. urticae*-infested leaves (Figure 3F–I, $P \geq 0.19$, binomial test).

Finally, we investigated the role of MeSA, (*E*, *E*)-TMTT, and 2-butanone in enabling *P. persimilis* to discriminate between the volatiles blends induced by its prey *T. urticae* and the nonprey caterpillar *S. exigua*. Because the choices of

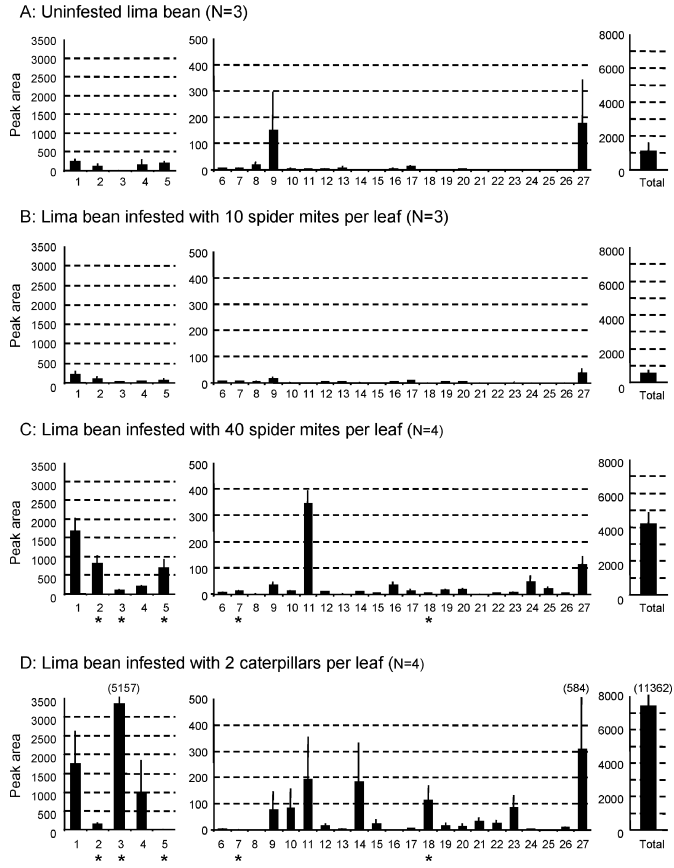

FIG. 1. Emission of volatiles by lima bean plants infested with different densities of *Tetranychus urticae*, or with *Spodoptera exigua*. Bars present the average emission of volatiles for *N* replicates for uninfested lima bean plants (A), lima bean plants infested with 10 (B) or 40 *T. urticae* per leaf (C), or 2 *S. exigua* per leaf (D). Error bars represent the standard error. Compound numbers indicate: (1) (*E*)-4,8-dimethyl-1,3,7-nonatriene, (2) (3*E*, 7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, (3) (*E*)-β-ocimene, (4) (*Z*)-3-hexen-1-ol acetate, (5) methyl salicylate, (6) nonanal, (7) decanal, (8) 1-penten-3-ol, (9) (*Z*)-3-hexen-1-ol, (10) hexyl acetate, (11) 2-butanone, (12) 3-pentanone, (13) 3-octanone, (14) 2-methylbutanal-*O*-methyl oxime, (15) 3-methylbutanal-*O*-methyl oxime, (16) ethyl salicylate, (17) limonene, (18) (*Z*)-β-ocimene, (19) (*Z*)-4,8-dimethyl-1,3,7-nonatriene, (20) 4,8,12-trimethyl-1,3,7,11-tridecatetraene-isomer, (21) β-caryophyllene, (22) *p*-mentha-1,3,8-triene, (23) indole, (24) unknown, (25) unknown, (26) unknown, (27) unidentified compounds. The quantity of compounds emitted by plants infested with 40 *T. urticae* (C) or 2 *S. exigua* (D) per leaf was analyzed with a Mann–Whitney *U* test; compounds that were emitted in significantly different amounts by these two treatments—$^{*}P < 0.05$.

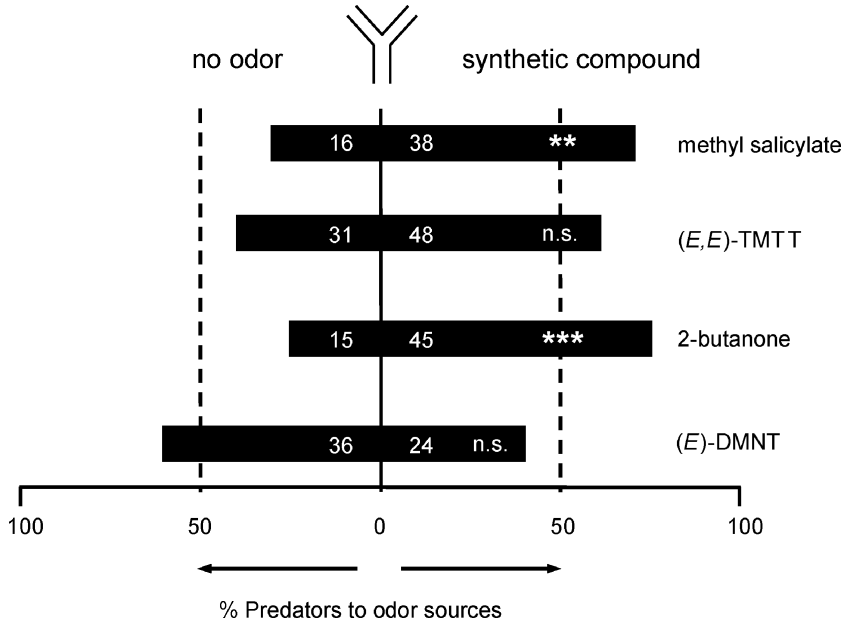

FIG. 2. Response of *P. persimilis* to the single synthetic compounds MeSA, (E, E)-TMTT, 2-butanone, and (E)-DMNT. Release rates were 10 μg/hr for MeSA, 0.5 μg/hr for (E, E)-TMTT, 4 mg/hr for 2-butanone, and 11 μg/hr for (E)-DMNT. Bars present the overall percentages of predatory mites choosing for each odor source; numbers in bars are the total numbers of predators choosing for each odor source. Choices between odor sources were analyzed with a two-sided binomial test (ns $P > 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

predatory mites between *T. urticae*-induced and *S. exigua*-induced volatiles did not differ before and after the test in which a synthetic volatile had been added to the *S. exigua*-induced volatiles ($P > 0.05$, GLM, predicting factors: replicate + odor source), we pooled the results of these two subexperiments. On average, 68% of the predators preferred the volatiles induced by *T. urticae* to those induced by *S. exigua* (Figure 4, $P \leq 0.002$, binomial test). After adding MeSA to the *S. exigua*-induced volatiles, as much as 78% of the predators preferred this combination to *T. urticae*-induced volatiles ($P < 0.001$, binomial test). (E, E)-TMTT had a similar effect: after adding (E, E)-TMTT to the *S. exigua*-induced volatiles, 61% of the predators preferred this combination to *T. urticae*-induced volatiles ($P = 0.03$, binomial test). Both MeSA and (E, E)-TMTT had a significant effect on the choice of *P. persimilis* between the volatiles from *T. urticae*-infested and *S. exigua*-infested lima bean leaves ($P < 0.001$, GLM). 2-Butanone, a compound emitted in similar amounts by both *T. urticae*-infested and *S. exigua*-infested lima

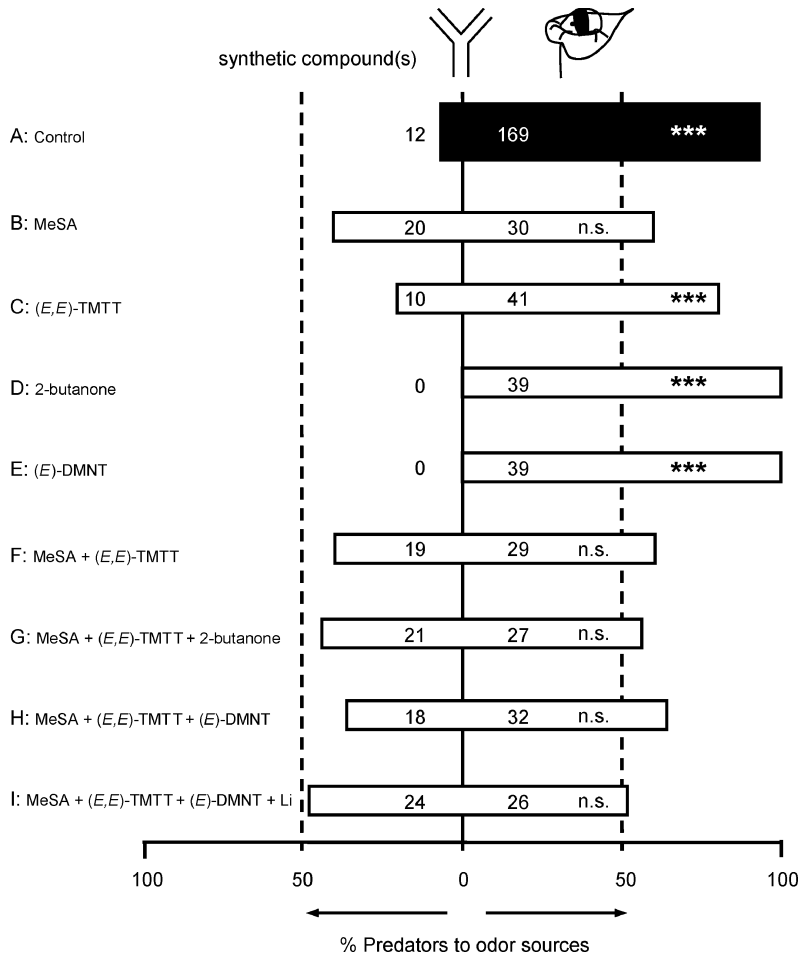

FIG. 3. The choices of predatory mites between the natural volatile blend induced by their prey in lima bean, and one to three synthetic compounds. The black bar presents the overall result of 18 replicates to test the attractiveness of the volatiles from *T. urticae*-infested lima bean leaves (40 spider mites per leaf) *vs.* no odor (A). The white bars present the choice of predatory mites between the volatiles from *T. urticae*-induced lima bean and: (B) MeSA, (C) (E, E)-TMTT, (D) 2-butanone, (E) (E)-DMNT, (F) MeSA + (E, E)-TMTT, (G) MeSA + (E, E)-TMTT + 2-butanone, (H) MeSA + (E, E)-TMTT + (E)-DMNT, (I) MeSA + (E, E)-TMTT + (E)-DMNT + the volatiles from four uninfested lima bean leaves (Li). Release rates were 10 μg/hr for MeSA, 0.5 μg/hr for (E, E)-TMTT, 4 mg/hr for 2-butanone, and 11 μg/hr for (E)-DMNT. Numbers in bars are the absolute numbers of predators choosing for each odor source. Choices between odor sources were analyzed with a two-sided binomial test (ns $P > 0.05$; $^{***}P < 0.001$).

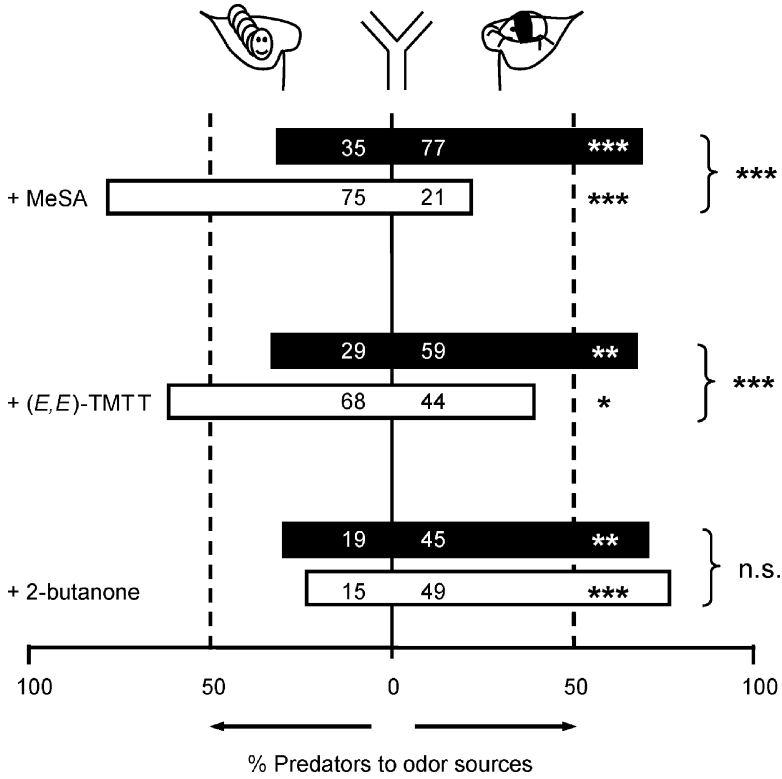

FIG. 4. The role of MeSA, (E, E)-TMTT, and 2-butanone in the choices of predatory mites between the volatiles from *T. urticae*-infested and *S. exigua*-infested lima bean plants. Herbivore densities were 40 *T. urticae* and 2 *S. exigua* per leaf. Release rates of synthetic volatiles were 10 μg/hr for MeSA, 0.5 μg/hr for (E, E)-TMTT, and 4 mg/hr for 2-butanone. Black bars present the choices between the volatiles from *T. urticae*-infested *vs*. *S. exigua* infested lima bean leaves; white bars present the choices between the volatiles from *T. urticae*-infested leaves *vs*. the combination of *S. exigua*-infested leaves and the synthetic compound indicated on the left side of the white bar. Numbers in bars are the total numbers of predators responding to each odor source. Choices between odor sources were analyzed with a two-sided binomial test. The effect of adding the synthetic volatile to *S. exigua*-induced volatiles was analyzed with a GLM, the significance of which is indicated behind the brackets (ns $P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

bean plants, did not have such an effect ($P = 0.41$, GLM). When 2-butanone was added to the *S. exigua*-induced volatiles from lima bean, the predators still preferred the volatiles induced by their prey *T. urticae* (77%, $P < 0.001$, binomial test).

DISCUSSION

This study shows that the predatory mite *P. persimilis* uses MeSA and (*E*, *E*)-TMTT to discriminate between volatile blends from lima bean induced by 40 *T. urticae* or 2 *S. exigua* per leaf (Figure 4). The role of MeSA in the searching behavior of *P. persimilis* is also supported by the loss of preference of the predators for the volatiles from *T. urticae*-infested bean when the single compound MeSA was offered as an alternative odor source (Figure 3). MeSA has been identified as an attractant for predatory arthropods in previous studies—for example, predatory mites, anthocorid bugs, and a green lacewing (Dicke et al., 1990; Scutareanu et al., 1997; James 2003; De Boer and Dicke, 2004a). Moreover, MeSA has been identified as the major determinant of the preference of *P. persimilis* when given a choice between the volatiles from *T. urticae*-infested and jasmonic acid-treated lima bean plants (De Boer and Dicke, 2004a). The effect of (*E*, *E*)-TMTT on the preference of *P. persimilis* for *T. urticae*-induced volatiles was less pronounced than that of MeSA (Figures 3 and 4). This might be explained by the difference in concentration between the two compounds: MeSA was released at a 20 times higher rate than (*E*, *E*)-TMTT. However, we previously showed that a quantitative difference (in the range of 0.2–20 μg) in the amount of MeSA does not affect discrimination by *P. persimilis* between two odor sources (De Boer and Dicke, 2004a). Moreover, 2-butanone was released at an even higher rate than MeSA and attracted the predators when offered vs. clean air, but it did not affect the discrimination by *P. persimilis* between *T. urticae*-induced and *S. exigua*-induced volatiles (Figure 4). This was expected because 2-butanone is emitted at similar levels by both *S. exigua* and *T. urticae*-infested lima bean plants (Figure 1). It is not yet known what the effect is of (*E*, *E*)-TMTT quantity on the foraging behavior of predatory mites.

In contrast to data presented by Dicke et al. (1990), (*E*)-DMNT did not attract *P. persimilis* in this study (Figure 2). This may be explained by a difference in starvation level of the predators. Dicke et al. (1990) used satiated predators, while we used 24 hr starved predators. We have done an experiment in which we tested 24 hr starved predators and 2 hr starved predators alternately in the olfactometer while offering (*E*)-DMNT in a capillary *vs.* an empty arm. The 2 hr starved predators were attracted to (*E*)-DMNT (78% of the predators chose for (*E*)-DMNT, $N = 41$, $P = 0.001$) while 24 hr starved predators were not attracted (60% of the predators chose for (*E*)-DMNT, $N = 52$, $P = 0.11$).

Our result may be surprising because MeSA and (*E*, *E*)-TMTT are not only commonly induced upon spider mite infestation in a range of plant species (e.g., Krips et al., 1999; Horiuchi et al., 2003; Van den Boom et al., 2004), but also in the same and other plant species upon infestation with different herbivore species (e.g., Scutareanu et al., 1997; Van Poecke et al., 2001; Fritzsche Hoballah et al., 2002). The commonness of these compounds may suggest that they are not reliable

indicators of the identity of the herbivore inducing the volatile emission. However, previous experience of predatory mites with specific volatiles most likely enables them to use such compounds in prey location (De Boer and Dicke, 2004b; De Boer et al., 2004). The predatory mites used in this study were reared on spider mites on lima bean and, thus, experienced large amounts of MeSA and (*E*, *E*)-TMTT, but for example, low amounts of (*E*)-β-ocimene. We recently showed that experience with spider mite-induced lima bean volatiles indeed influences the ability of *P. persimilis* to discriminate between *T. urticae*-induced and *S. exigua*-induced lima bean volatiles (De Boer et al., 2004). Similar learning mechanisms have been demonstrated before in other natural enemies of herbivorous arthropods, such as parasitoid wasps (e.g., Turlings et al., 1993; Vet et al., 1995). Moreover, predatory mites can learn to respond to MeSA (De Boer and Dicke, 2004b). Phenotypic plasticity or learning enables natural enemies to specialize temporarily on specific compounds that signal the presence of suitable prey herbivores, while in the long-term it enables the species to use a broad range of volatile compounds for this purpose.

One might argue that besides using volatiles induced by the prey herbivore, natural enemies could also use compounds induced by the nonprey herbivore in order to avoid plants with unsuitable herbivores. However, compounds that are induced by nonprey herbivores in certain plant species might be induced by suitable prey herbivores in other plant species. For example, (*E*)-β-ocimene is induced in a large amount by *S. exigua* in lima bean plants (Figure 1) but it is also induced in large amounts in cucumber plants infested with spider mites (e.g., Takabayashi et al., 1994; De Boer, 2004). Natural enemies might be able to use specific compounds to avoid plants infested with nonprey herbivores after experiencing such compounds in the absence of prey (Papaj et al., 1994; Vet et al., 1998). We recently showed that exposing predatory mites to *S. exigua*-induced volatiles in the absence of food did not have a significant effect on the discrimination behavior of *P. persimilis* (De Boer et al., 2004). This indicates that even after experience, the volatiles induced by the nonprey herbivore were less important in enabling predatory mites to discriminate than the volatiles induced by the prey herbivore.

An important issue that remains to be addressed is the ecological relevance of our finding. In a natural environment, conditions might differ from our laboratory setup in such a way that predators cannot use MeSA or (*E*, *E*)-TMTT to locate plants infested with suitable prey herbivores. For example, MeSA and (*E*, *E*)-TMTT might not always be induced differentially between prey and nonprey herbivores. First, this could be caused by herbivore density. Females of *S. exigua* oviposit clusters of 20–50 eggs, and caterpillars feed gregariously (Smits et al., 1986). Horiuchi et al. (2003) showed that in lima bean certain herbivore-induced volatiles increase with *S. exigua* density [e.g., (*E*)-β-ocimene], whereas other compounds do not (e.g., MeSA). However, at higher densities of *S. exigua*, the

predatory mite *P. persimilis* avoids the volatiles from caterpillar-infested plants. Volatiles from caterpillar feces mediate this avoidance (Shimoda and Dicke, 1999). Discrimination based on plant volatiles seems, therefore, less "necessary" at higher levels of *S. exigua* infestation. Second, different plant species and species of non-prey herbivore could lead to volatile blends that do not differ in the amounts of MeSA or (*E*, *E*)-TMTT. Apart from the presence of specific compounds, the ratios between compounds in the volatile blends may play a role. Quantitative differences in volatile blends have been found in a range of plant-herbivore combinations, including infestation of maize, tobacco, or cotton with the caterpillars *Helicoverpa zea* and *Heliothis virescens* (DeMoraes et al., 1998), infestation of apple with the spider mites *T. urticae* or *Panonychus ulmi* (Takabayashi et al., 1991), and infestation of cabbage with the caterpillars *Pieris rapae* or *Plutella xylostella* (Shiojiri et al., 2001). Carnivorous arthropods can indeed discriminate among plants infested with these different herbivore species, which suggests a role for the ratios between compounds in carnivore foraging behavior.

Despite these considerations, our experiments provide the first evidence that specific compounds from herbivore-induced volatile blends can play an important role in the foraging behavior of natural enemies of herbivorous arthropods. Although at this point this conclusion can only be drawn for the specific tritrophic system and herbivore densities that we used, this is a novel finding that should inspire studies on unraveling the volatile compounds that mediate interactions in other tritrophic systems.

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SELECTION OF *Nothofagus* HOST TREES BY THE APHIDS *Neuquenaphis staryi* AND *Neuquenaphis edwardsi*

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Abstract—Leaf volatiles were collected from three *Nothofagus* species growing in close proximity in Los Ruiles National Reserve, Chile. The volatile preparation from leaves of *No. alessandrii* were attractive to the specialist aphid, *Neuquenaphis staryi*, but not to the generalist aphid, *Ne. edwardsi*, while the volatile preparations of *No. dombeyi* and *No. glauca* were attractive to *Ne. edwardsi*, but not to *Ne. staryi*. This reflects the pattern of aphid/host-plant associations. α-Agarofuran was found to occur in all leaf volatile preparations and was shown by electroantennography and olfactometry to be attractive for both *Neuquenaphis* spp., suggesting it may be the *Nothofagus* host-recognition factor for *Neuquenaphis*. The factor(s) mediating *Ne. staryi*'s specialization on *No. alessandrii* remain to be identified.

Key Words—*Nothofagus, Neuquenaphis*, leaf volatiles, α-agarofuran, olfactometry, electroantennography.

INTRODUCTION

There is ample evidence that the host-selection behavior of herbivorous insects is mediated primarily by secondary plant chemicals, although cues at other trophic levels, such as from natural enemies or competitors, may also be important in certain cases (Dicke, 2000). Southern beeches (*Nothofagus* spp.) in Chile are parasitized by the native aphid genus, *Neuquenaphis*. This genus is quite diverse and exhibits a wide range of specificity within *Nothofagus* hosts, from the specialist *Ne. staryi*, which is found only on *No. alessandrii*, to the generalist *Ne. edwardsi*, which has been found on eight host tree species (Quiroz et al., 1999, and

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unpublished results). The pattern of association between *Neuquenaphis* and *Nothofagus* probably arises from the capacity of *Neuquenaphis* to respond to chemical and physical features of the host plants. Selection and acceptance by aphids of their host plants is a complex and highly integrated process that occurs in four successive stages (Niemeyer, 1990). The first of these stages (landing on the plant) involves chemical attraction by volatiles emanating from the surface of the leaf. At each of the other stages, the aphid may find plant features that induce it to continue on the plant or to leave.

In earlier work (Quiroz et al., 1999), the distribution of four *Neuquenaphis* species on *Nothofagus* was reported, and a preliminary analysis of the leaf volatiles of six *Nothofagus* species was presented. A major component in the volatile profile of *No. glauca* was identified as the terpene, α-agarofuran. This compound was attractive to alates of *Ne. sensoriata*. We wish to clarify here that the alates of *Ne. sensoriata* used in that study, were misidentified (C. C. Ramírez, personal communication) and that the aphid recorded as *Ne. sensoriata* in Quiroz et al., 1999, was, in fact, *Ne. edwardsi*. Therefore, the results reported in Quiroz et al., 1999, relate to the generalist aphid, *Ne. edwardsi* that may find its host plants by attraction to α-agarofuran.

Los Ruiles National Reserve, 200 km south of Santiago, is an isolated coastal pocket where the last remaining stands of *No. alessandrii* are to be found. It occurs mixed with *No. glauca* and *No. dombeyi*. As reported previously (Quiroz et al., 1999), *Ne. staryi* was found only on *No. alessandrii* and not on the other two neighboring species in this reserve, whereas *Ne. edwardsi* was found on both *No. glauca* and *No. dombeyi* and not on *No. alessandrii*. There is almost certainly a specific attraction for *Ne. staryi* to *No. alessandrii* and an ability for it to survive on this plant. *No. alessandrii* has few associated insects and its leaf extracts show toxicity in several bioassays (Russell et al., 2000). By the same token, *Ne. edwardsi* must be attracted to, and have an ability to survive on, *No. glauca* and *No. dombeyi* rather than on *No. alessandrii*.

In order to extend our previous work (Quiroz et al., 1999) and to confirm the primary attraction of *No. alessandrii* for alates of *Ne. staryi*, and *No. glauca* and *No. dombeyi* for alates of *Ne. edwardsi*, we undertook further work with the leaf volatiles of *No. alessandrii*, *No. glauca*, and *No. dombeyi* from the Los Ruiles National Reserve.

METHODS AND MATERIALS

Plants and Insects. Both plants and aphids were collected from Los Ruiles National Reserve (32°49′ S: 72°31′ W), Chile. Leafy stems (0.5 m) were cut from branches of *No. alessandrii* Ep., *No. glauca* (Phil.) Krasser, and *No. dombeyi* (Mirb.) Oerst., and stored in a cool-box during transportation to the laboratory.

Alates of *Ne. staryi* and *Ne. edwardsi* were collected from leaves of their host tree and kept on leaves in suitable tubes and at cool temperatures (4°C) until required.

Entrainment of Leaf Volatiles. The cut end of each stem was sealed with Teflon tape and the stems (4) placed into a 10-l bell-jar. Air was drawn through the jar by an oil-free pump at 1 l/min for 20 hr, and was purified by passage through an inlet filter containing activated charcoal and molecular sieves. The air exited from the jar through a Porapak Q (50/80 mesh, 30 mg) trap that entrained the leaf volatiles. These were desorbed from the Porapak Q with distilled dichloromethane (2 ml) and concentrated (200 μl) under a stream of nitrogen to give an extract containing the leaf volatiles. Several entrainments were carried out to acquire sufficient volatile concentration for the bioassays.

Analysis of Volatiles. Aliquots (1 μl) of the concentrated volatile extracts were analyzed by GC–MS. A capillary Supelco SPB-5, GLC column (30 m $\times$ 0.25 mm ID) was directly coupled to a mass detector with an integrated data system (GC model HP-56690, MD model HP-5972). Ionization was by electron impact at 70 eV and 280°C. The GC oven was programmed to remain at 40°C for 10 min and then increase at 5°C/min to 250°C. The helium carrier gas flow was 1 ml/min. The GC peaks of each volatile extract were identified by comparison of their retention times and mass spectra with commercial standards. When these were not available, mass spectra were compared with a computerized library data-base (NIST98).

Electroantennogram Detection (EAD). Aliquots (2 μl) of volatile extracts were injected into a Shimadzu GC equipped with a HP-5 MS (similar liquid phase and column dimensions as above) and a 1:1 effluent splitter that allowed simultaneous FI detection and electroantennogram detection. Nitrogen was used as the carrier gas (1 ml/min), and the oven was programmed as above. The outlet for the EAD was held in a humidified air-stream flowing at 5 ml/sec over a *Neuquenaphis* antennal preparation. A glass microcapillary indifferent electrode filled with Ringer solution and grounded with a silver wire was inserted into the aphid's head close to an antenna. A similar recording electrode connected to an amplifier was positioned in the distal end of the antenna. The signal was stored and analyzed on a PC with the EAD program from Syntech, The Netherlands.

Olfactometry. Behavioral studies were performed in an olfactometer as described by Pettersson (1970). The olfactometer consisted of a quadratic arena permeated by air (100 ml/min) coming from the stretched corners (arms) and drawn out through a hole above its center. Two glass tubes containing the same volatile extract were connected to the end of two consecutive arms and two tubes containing the competing stimulus or solvent control were connected to the other two arms. The observation arena was divided into four zones and one indifferent zone in the center. An aphid was placed onto the center of the arena and the time it spent in each arm was recorded during 15 min. Each experiment was replicated at least seven times and the results analyzed by nonparametric statistics (Wilcoxon

one-tailed rank-sum test for two groups). The mean times spent in each zone containing the volatiles sources were compared. Authentic α-agarofuran was obtained from *No. glauca* and characterized as previously described (Quiroz et al., 1999).

RESULTS AND DISCUSSION

The leaf volatiles from the three species of *Nothofagus* leaves were entrained from the head-space of several small leafy branches cut from the trees. This method differed from the preferred field-trapping method used in the previous study (Quiroz et al., 1999), but it enabled longer trapping times from multiple collections and gave greater yields of entrained volatiles for bioassay. *No. alessandrii* gave the poorest yield of volatiles with *No. dombeyi* giving 2×'s, and *No. glauca* 4×'s the yield of total volatiles on the basis of a common internal standard. This meant that while four trappings of *No. dombeyi* and *No. glauca* were sufficient for testing with aphids, several collections of *No. alessandrii* were combined to provide sufficient material. Each volatile preparation was analyzed by GC–MS and, although the same compounds were almost always present for a particular species, yields of the individual components varied markedly.

The identity and relative abundance of the entrained leaf volatiles collected from the three *Nothofagus* species are shown in Table 1, which represents a typical GC–MS profile of leaf volatiles collected in late November/early December. GC–MS traces showed typical atmospheric contaminants, such as alkanes and substituted benzenes, reflecting the long collection times employed, and in spite of having a carbon/molecular sieve filter on the inlet of the head-space apparatus. These contaminants and the siloxanes originating from the GC column liquid phase, have been eliminated from Table 1. All the compounds identified in the previous work (Quiroz et al., 1999) were found in this study, but we were able to identify a greater number (48 as opposed to 21) occurring in one or more species. This is a reflection of the methods used and the longer trapping times employed. Over the three species, 66 peaks were observed in the GC: 22 were identified with authentic compounds, 26 were provisionally identified from a mass spectral library match, 18 peaks remain unidentified. The identified compounds fall into three categories. Common monoterpenes found as constituents of many leaf volatiles (Knudsen et al., 1993), esters and aldehydes, also found in many leaf volatiles, and a significant group of sesquiterpenes in which cadinane and eudesmane structural-types predominate.

Compounds that are common to the three *Nothofagus* species are: α-pinene (**3**), camphene (**4**), sabinene (**6**), α-copaene (**33**), (*E*)-β-farnesene (**44**), germacrene D (**48**), δ-cadinene (**56**), α-agarofuran (**57**), and γ-eudesmol (**64**). *No. glauca* gave relatively high yields of the monoterpenes, α-pinene and sabinene, whereas *No. dombeyi* gave high yields of α-copaene. Compounds found only in *No. alessandrii*

TABLE 1. COMPOSITION OF LEAF VOLATILES FROM THREE *Nothofagus* SPECIES

	Compound	Identification method[b]	Retention time (min)	Relative areas[a] (%)		
				N. alessandrii	*N. glauca*	*N. dombeyi*
1	Thujene	B	12.54			0.9
2	Tricyclene	B	13.01	2.0		2.8
3	α-Pinene	A	13.79	2.2	18.4	2.9
4	Camphene	A	14.56	0.1	0.8	5.2
5	Benzaldehyde	A	15.23	0.1		
6	Sabinene	B	16.02	8.0	52.1	1.3
7	Phenol	A	16.87	0.1		
8	6-Methyl-5-hepten-2-one	A	16.90	0.1		
9	(*Z*)-3-Hexenyl acetate	A	17.85	5.0	0.5	
10	α-Terpinene	B	18.11		0.6	
11	Hexanyl acetate	A	18.14	0.6		
12	(*E*)-2-Hexenyl acetate	A	18.23	0.8		
13	*p*-Cymene	A	18.46	1.8	1.2	
14	Limonene	A	18.68	1.7	0.9	
15	(*E*)-Ocimene	B	19.59		0.2	0.6
16	γ-Terpinene	A	19.95		1.0	
17	β-Terpineol	B	20.27		1.2	
18	Terpineolene	A	21.20		0.3	
19	Nonanal	A	21.79	2.0		
20	Unknown 1 (M, 150)		22.76	9.7	21.0	
21	Camphor	A	23.09		0.1	0.6
22	Isocamphopinone	B	24.14		0.1	

TABLE 1. CONTINUED

	Compound	Identification method[b]	Retention time (min)	Relative areas[a] (%)		
				N. alessandrii	*N. glauca*	*N. dombeyi*
23	4-Ethylbenzaldehyde	A	24.27	0.1		
24	4-Terpineol	A	24.28		0.2	
25	Unknown 2 (M, 146)		24.68	0.2		
26	Unknown 3 (M, 152)		24.86	0.1		
27	Decanal	A	25.37	1.3	0.5	
28	4-Terpineol acetate	B	25.71		0.5	
29	Unknown 4 (M, 136)		28.47	0.6		
30	α-Cubebene	A	29.73	2.5		0.3
31	α-Muurolene	B	30.23			1.0
32	Unknown 5 (M, 174)		30.35	2.8		
33	α-Copaene	A	30.59	0.6	1.4	35.3
34	Unknown 6 (M, 204)		30.77	0.7	0.4	
35	β-Santalene	B	30.91	1.2	0.2	
36	α-Cedrene	A	30.59	0.2		
37	Caryophyllene	A	31.70		1.0	2.5
38	α-Humulene	B	31.75	0.6		
39	α-Santalene	B	31.76		0.4	
40	Unknown 7 (M, 204)		32.02		0.2	
41	β-Bergmantene	B	32.14	0.2	0.2	
42	α-Guaiene	B	32.25	1.3		
43	Unknown 8 (M, 204)		32.41		0.1	0.4
44	(*E*)-β-Farnesene	B	32.68	0.3	0.9	3.3

TABLE 1. CONTINUED

	Compound	Identification method[b]	Retention time (min)	Relative areas[a] (%)		
				N. alessandrii	*N. glauca*	*N. dombeyi*
45	Aromadendrene	B	32.86		0.8	4.2
46	Unknown 9 (M, 220)		33.15	0.2		0.3
47	α-Curcumene	B	33.29	0.7		0.7
48	Germacrene D	B	33.43	2.6	5.3	1.5
49	β-Selinene	B	33.53		0.4	
50	γ-Cadinene	B	33.69			0.5
51	α-Selinene	B	33.75		0.5	
52	γ-Muurolene	B	33.87		0.6	
53	α-Farnesene	B	34.00	3.2	0.3	
54	Unknown 10 (M, 204)		34.15	9.7		
55	Unknown 11 (M, 204)		34.24		0.4	
56	δ-Cadinene	B	34.47	1.2	0.6	12.8
57	α-Agarofuran	A	35.08	4.7	2.0	2.3
58	Unknown 12 (M, 220)		35.32	1.4		
59	Nerolidol	B	35.36		1.6	1.2
60	Unknown 13 (M, 220)		35.45	4.7		
61	Unknown 14 (M, 218)		35.67	1.0		
62	Unknown 15 (M, 220)		35.84	2.1		
63	Unknown 16 (M, 222)		36.62		1.6	1.2
64	γ-Eudesmol	B	36.95	1.0	0.9	8.7
65	Unknown 17 (M, 222)		37.40		0.9	0.4
66	Unknown 18 (M, 222)		38.16	0.7		

[a] Calculated as a percentage of the total volatile peak area for that plant.
[b] A = comparison with authentic compounds; B = GC–MS comparison with the library database (NIST 98) with a similarity index >95%.

were: benzaldehyde (**5**), phenol (**7**), 6-methyl-5-hepten-5-one (**8**), hexanyl acetate (**11**), (*E*)-2-hexenyl acetate (**12**), nonanal (**19**), 4-ethylbenzaldehyde (**23**), α-cedrene (**36**), α-humulene (**38**), α-guaiene (**42**), and several unknowns. Aldehydes and esters seem to be common in *No. alessandrii*, while a greater variety and yield of monoterpenes seem to occur in the volatile profile of *No. glauca*.

Results of olfactometry studies with solutions of entrained volatiles against *Ne. staryi* and *Ne. edwardsi* are shown in Figure 1. When *Ne. staryi* was given pair-wise choices between volatiles of *No. alessandrii* and volatiles of either *No. glauca* or *No. dombeyi* or of a solvent control, the aphid preferred to move toward the olfactometer arms containing the *No. alessandrii* volatiles, indicating that there is a specific attractant(s) for *Ne. staryi* in the leaf volatiles of *No. alessandrii*. When *Ne. staryi* was presented with a choice between volatiles of *No. glauca* and *No. dombeyi*, and between each of these volatile preparations and solvent, the aphid moved between both arms spending a similar time in each. This indicates that these volatile blends were no more attractive than the solvent controls, eliciting a neutral response from the aphid. When *Ne. edwardsi* alates were offered the same pair-wise choices, the aphid moved toward the arms containing either volatiles of *No. glauca* or *No. dombeyi*, indicating it was attracted to volatiles of these plants rather than the volatiles of *No. alessandrii*. When presented with a choice of *No. alessandrii* volatiles and solvent, it spent equal time in each arm unable to make a preference decision. Given *Ne. edwardsi*'s generalist nature and its known attraction for α-agarofuran, a component in *No. alessandrii* volatiles, the volatile mixture from *No. alessandrii* either lacks an additional component(s) that will determine preference or there is a component(s) in the mixture that is deterring the aphid from settling at the *No. alessandrii* source.

Olfactometry studies support field observations for host associations of *Ne. staryi* and *Ne. edwardsi*, and indicate that the leaf volatiles are a source of primary host attraction. In order to delineate this source of attraction in the host plant volatiles, we carried out some experiments with an electroantennogram used as a secondary detector (EAD) to a GC. Achieving functional antennal preparations with aphids is known to be difficult, due to there being few olfactory sensilla and low response voltages (Pickett et al., 1992), and we had limited success with this technique. We were unable to achieve a successful functioning antennal preparation of *Ne. edwardsi*, but a preparation with an antenna of *Ne. staryi* gave two clear signals with *No. alessandrii* volatiles, which we were able to correlate with peaks in the GC–MS. One signal corresponded to the peak for α-agarofuran (**57**). We were able to confirm this EAD response using authentic α-agarofuran with *Ne staryi*; an EAD signal coincided with the FID peak for α-agarofuran. The second signal corresponded to compound **54**, but we have been unable to identify this compound (unknown 10) as an attractant for *Ne. staryi*.

In the olfactometer bioassay, alates of *Ne. staryi* were attracted by authentic α-agarofuran compared with the solvent control (Table 2). Both *Ne. staryi* and *Ne.*

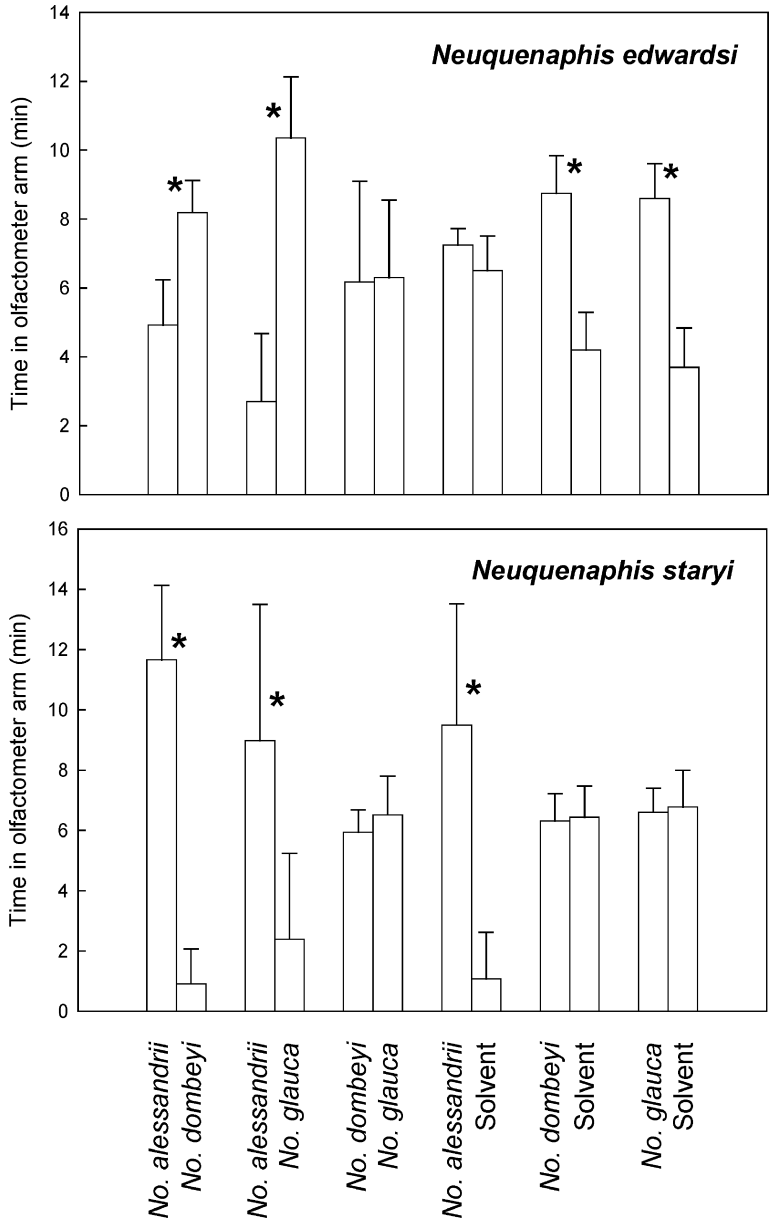

FIG. 1. Preferences of *Neuquenaphis edwardsi* and *Neuquenaphis staryi* for different stimuli offered pair wise in an olfactometer. Asterisks denote significant differences ($P < 0.05$, Wilcoxon one-tailed rank-sum test for two groups).

TABLE 2. RESPONSE OF *Neuquenaphis* INTRODUCED SINGLY INTO AN OLFACTOMETER AND EXPOSED TO α-AGAROFURAN

	Time spent (min)[a]		
	Solvent[b]	10 μg of α-agarofuran	P^c
Ne. staryi alatae	3.2 ± 2.4	8.1 ± 4.1	0.02
Ne. edwardsi alatae[d]	4.9 ± 1.7	8.0 ± 1.1	0.01

[a]Mean $\pm$ standard error.
[b]Dichloromethane for *Ne. staryii* and hexane for *Ne. edwardsi*.
[c]Wilcoxon one-tailed rank-sum test for two groups.
[d]Data from Quiroz et al., 1999.

edwardsi, a specialist aphid and a generalist aphid, respectively, are attracted to α-agarofuran. α-Agarofuran is not a common terpene, but with this and previous work, we have now shown its occurrence in five *Nothofagus* species. This raises the possibility that α-agarofuran may be found in most *Nothofagus* and may be the principal host-recognition factor for *Neuquenaphis*. If that is the case, then the question remains, what factor(s) determines *Ne. staryi's* selection of *No. alessandrii* as its only host plant and *Ne. edwardsi's* avoidance of this plant? This factor(s) may fulfill both functions, attracting *Ne. staryi* on the one hand and repelling *Ne. edwardsi* on the other, although the presence or absence of attractants in the volatile blends may be the more important principle. Del Campo et al. (2003), suggested that it was the recognition of host-specific chemicals rather than the avoidance of deterrents that determined the feeding behavior of the pea aphid, *Acyrthospiphon pisum*. Compound **54**, which occurs only in *No. alessandrii*, may be the other factor that allows *Ne. staryii* to recognize its host plant, but it has not been possible to confirm this. The leaf chemistry of *No. alessandrii* is quite different from the other Chilean *Nothofagus* (although it has similarities to the New Zealand *No. fusca*) and we have found the phytoalexin, pinosylvin, occurring only in *No. alessandrii* leaf extracts (Russell et al., 2000). However, we could not find pinosylvin or any related phenolics in the head space volatiles from leaves of *No. alessandrii*, ruling out the possibility that such phenolics may be the unique host-recognition factors for *Ne. staryi*. Olfactometer experiments with benzaldehyde, phenol, 4-ethylbenzaldehyde, nonanal, 2-hexenyl acetate, and hexanyl acetate, which occur only in *No. alessandrii* volatiles, also indicated that these compounds were unlikely attractants to *Ne. staryii* or repellants to *Ne. edwardsii*.

Primary host selection behavior by herbivorous insects is mediated mainly by plant volatiles. In this study, we have shown that leaf volatile chemicals (constitutive or induced) can be collected and influence the host recognition behavior of a specialist and a generalist *Neuquenaphis* in a manner that reflects the observed plant–insect associations in the natural environment. It appears that the principal

host-recognition factor for *Neuquenaphis* on *Nothofagus* is α-agarofuran and that other factors play a role in the specialization of *Ne. staryi*.

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CHEMICAL COMPOSITION OF CORN LEAF ESSENTIAL OILS AND THEIR ROLE IN THE OVIPOSITION BEHAVIOR OF *Sesamia nonagrioides* FEMALES

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Abstract—The chemical composition of the volatile oils collected by steam distillation from leaves of seven corn hybrids and their effect on the oviposition behavior of *Sesamia nonagrioides* females was studied. Samples of the volatile oils from each of the hybrids were analyzed by capillary gas chromatography-mass spectrometry (GC-MS) and several major compounds were identified. The major compound found in all seven hybrids was 3,7,11,15-tetramethyl-2-hexadecen-1-ol (phytol), ranging from 38.3 to 64.9% of the total quantity. Compounds detected in significant proportions include (*Z*)-3-hexenol (3.1 to 8%), nonanal (4.9 to 14.5%), pentadecanal (1.8 to 5.8%), neophytadiene (5.5 to 12.9%), (*Z*)-3-hexenyl acetate (2.5 to 8.9%), and an analogue of 2,4-dihydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3-(4*H*)-one (DIMBOA) (2.4 to 9.3%). The analysis showed no qualitative differences among the chemicals identified while quantitative differences were detected. Among the volatile oils, the significant difference was in the quantity of aldehydes present. In two-choice bioassays, filter paper sticks treated with volatile oils containing higher quantity of aldehydes received fewer eggs than those with lower aldehyde quantity. Bioassays with synthetic aldehydes of a chain length C9–C14 confirmed the above results.

Key Words—*Sesamia nonagrioides*, corn hybrids, oviposition behavior, principal component, leaf volatile oils, aldehydes.

INTRODUCTION

Studies on chemical interaction between herbivores and plants have established that plant secondary metabolites play a significant role in host selection by insects (Miller and Strickler, 1984; Feeny, 1992; Städler, 1992; Renwick and Chew, 1994;

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Honda, 1995). The final decision by the female to oviposit or not, actually depends on the balance of opposing positive and negative cues evoked by phytochemicals that determine whether a plant is accepted or rejected (Huang and Renwick, 1993; Renwick and Chew, 1994).

Chemicals in corn plant extracts that stimulate or deter oviposition in *Ostrinia nubilalis* and *Chilo partellus* (Lepidoptera: Pyralidae) have been reported (Dittrick et al., 1983; Derridj et al., 1986, 1992; Lupoli et al., 1990; Udayagiri and Mason, 1995, 1997; Binder, 1999; Varshney et al., 2003). The chemical composition of volatile oils obtained by steam distillation from corn kernels, husks, silk, tassels, and leaf headspace volatiles collected on Tenax have been analyzed (Buttery et al., 1978, 1980; Flath et al., 1978; Buttery and Ling, 1984). All these studies suggest that corn secondary metabolites affect the oviposition behavior of lepidopteran corn pests.

Sesamia nonagrioides (Lef) (Lepidoptera: Noctuidae) is a serious corn pest in the Mediterranean countries; it is considered as oligophagus and attacks plants of the *Poaceae* family, with corn being its major host. Corn cultivars that are resistant to *S. nonagrioides* have been described, but the defensive mechanisms involved are not yet determined (Butrón et al., 1998; Velasco et al., 1999).

Konstantopoulou et al. (2002) demonstrated female oviposition preference on plants of certain corn hybrids, as well as on artificial substrates treated with leaf secondary metabolites extracted in pentane, or with leaf volatile oils collected in diethyl ether after steam distillation.

This paper reports on the chemical composition of the steam-distilled-volatile oils from the leaves of corn hybrids that are preferred by *S. nonagrioides* females vs. hybrids that are not preferred. The chemical profile of the volatile oils was correlated to the egg laying behavior of *S. nonagrioides*. The effect on oviposition behavior of *S. nonagrioides* females of the natural and synthetic aliphatic aldehydes is also discussed.

METHODS AND MATERIALS

Insects. Adults of *S. nonagrioides* used for behavioral tests were obtained from a laboratory colony that originated from a natural population collected at the pupal stage. The larvae were reared on artificial diet (Tsitsipis et al., 1983) for 3–4 generations and kept at $26 \pm 1^{\circ}$C with a 16L:8D regime and relative humidity of $65 \pm 5\%$. Adults were fed with sugar–water solution 10%.

Corn Plants. Five corn (*Zea mays*) hybrids, namely 33A14, 33R87, 3211, 31B13, and 3283W, provided by Pioneer Co (Pioneer Hi-Bred International, Des Moines, IA) and two Greek corn hybrids (Dias and Aris) purchased from the local market were used. Plants from seeds of the offspring of the commercially available hybrid Pioneer Konstanza (a receptive hybrid) were used as control. The 33A14 and 31B13 hybrids contain the YieldGard gene Cry1A(b) from

Bacillus thuringiensis, which provides enhanced resistance to lepidopteran pests. YieldGard is a trademark and used under license from Monsanto Co. Corn seeds were planted in small pots, in a green house under $25 \pm 1^{\circ}$C and relative humidity of $65 \pm 5\%$. For the behavioral tests and collection of volatile oils, plants were used 30 days after seeding, when their height was ca. 40 cm. At that developmental stage, females start to deposit eggs in the field.

C*ollection of Corn Leaf Volatile Oils*. The apparatus and method used to collect leaf volatile oils have been previously described (Konstantopoulou et al., 2002). Corn leaves (25 g) were steam distilled for 4 hr. The ether layer of each distillation was dried over magnesium sulphate, concentrated to 10 ml under a gentle stream of nitrogen, and stored at -20°C for bioassays or further analysis. Three replicates were carried out.

Gas Chromatography-Mass Spectrometry. One milliliter of the steam distillation extracts was reduced to 100 μl under a gentle stream of nitrogen. Prior to GC-MS analysis, 50 μg of *n*-tetradecane were added as internal standard to compensate for any variability introduced during the GC-MS analytical procedure. Gas chromatography-mass spectrometry analyses were carried out on an Hewlett Packard 5890 Series II gas chromatograph interfaced to a Fisons VG Trio 1000 (Manchester M23 9BE, UK) quadrupole mass spectrometer. Electron impact ionization was used, with electron energy of 70 eV and a trap current of 200 μA. All extracts were chromatographed on a 60 m $\times$ 0.25 mm (i.d.) $\times$ 0.1 μm film thickness DB-5 column (J & W Scientific, Folsom, CA, USA). The oven temperature program was 50°C for 2 min, then 5°C/min to 250°C, hold for 1 min, then 2°C/min to 280°C and hold for 50 min. Helium was used as carrier gas at a flow rate of 1 ml/min. Splitless injections were made (1 μl) at an injector temperature of 250°C and a splitless period of 90 sec.

Analysis of Chromatographic Data. Chromatogram peaks were identified using Wiley's mass spectral libraries and by comparing their chromatographic and mass spectrometric data with that of available synthetic standards. Peak areas were integrated using the MassLynx ver. 2.3 Mass Spectrometry Data Handling System (Micromass, Cheshire, UK). The ratio of each compound to internal standard peak response served as the analytical parameter. All results were normalized to the unit of the mass of leaves used for steam distillation, and are expressed in arbitrary units as the percentage of the proportion of the individual peak response to that of the internal standard divided by the mass of leaves extracted.

Bioassays for Ovipositional Response. Behavioral bioassays were conducted according to previously described methods (Konstantopoulou et al., 2002). Plants of the seven hybrids, the corresponding volatile oils, and synthetic aldehydes were tested.

Plants. In each of the seven cages (80 $\times$ 60 $\times$ 60 cm), one pot with a plant of each hybrid and one pot with a control plant were placed. One gravid 2-d-old female was released in each cage, and the number of eggs deposited per plant

was recorded 48 hr later. Data on each hybrid were collected from 15 replications. The experiments were conducted in a room, maintained at 26 ± 1°C and relative humidity of 65 ± 5% at a 16L:8D regime.

Volatile Oils. The oviposition preference or deterrence of females to the volatile oils collected from each hybrid was also examined in a two-choice bioassay. Filter paper sticks were used as oviposition substrates. In each cage (30 × 30 × 30 cm), one gravid female was released 2 hr before the onset of the dark phase. Two sticks were baited, one with 500 μl (equivalent to 500 mg of leaves) of the volatile oils of each hybrid and one with 500 μl of solvent. After solvent evaporation, the sticks were introduced into the cages 1 hr following the dark phase. The number of eggs deposited was recorded at the end of the dark phase. Data on volatiles for each hybrid and the controls were collected from 15 replications.

Synthetic Aldehydes. Female oviposition deterrence was examined using authentic samples of octanal (99% purity), nonanal (95% purity), decanal (95% purity), (Aldrich Chem. Co., Milwauke, WI, USA), and tetradecanal and pentadecanal 95 and 93% purity, respectively (provided by NPP S.A., Noguères, France). Five milligrams of each of the synthetic samples were diluted in 5-ml hexane. An amount of 500 μl of each solution containing 0.5 mg of the aldehyde were pipetted onto the filter paper sticks. The same volume of solvent was pipetted onto another stick. Sticks were left at room temperature for 15 min and then introduced into the cages where one gravid female was released 1 hr following the dark phase. For each sample examined, 10 replicates were carried out. The number of eggs deposited was recorded at the end of the dark phase.

Statistics. The mean number of eggs deposited on each plant and the filter paper sticks baited with each of the volatile oils or the solutions of the synthetic aldehydes was compared with the mean number of eggs deposited on the corresponding controls by a paired *t* test.

To investigate any association of the volatile oils chemical profile and oviposition preference, data for all identified compounds, were subjected to Principal Component Analysis (PCA). An advantage of PCA is that the original data can be transformed into linear combinations of an underlying set of hypothesized or unobserved components (Principal Components, PCs). PCA decomposes the covariance structure of the dependent variables into orthogonal components by calculating the eigenvalues and eigenvectors of the data covariance matrix. Eigenvectors or coefficients relate the specific association between principal components and original data. Therefore, it is necessary to find the coefficients, which will approximate the relationship between the original data and the underlying principal components (Jolliffe, 1986).

An index of oviposition (OI) for each of the hybrids tested and their volatile oils was calculated according to the formula described by Udayagiri and Mason (1997) and Konstantopoulou et al., (2002). The OI takes positive or negative

values indicating hybrids and/or volatile oils that are preferred by females for oviposition (positive values) and those that are not preferred (negative values).

To test if there is a linear association among the two OIs (plants and volatile oils) and the coefficients extracted from PCA principal components, the Pearson's correlation coefficients were computed. The Pearson correlation coefficient measures the strength and direction of a linear relationship between the X and Y variables, the sign of the coefficient indicates the direction of the relationship, and its absolute value indicates the strength; larger absolute values indicating stronger relationships. The statistical analyses were conducted with the SPSS 8.0 for Windows software.

RESULTS

Oviposition Preference–Plants and Volatile Oils. Two-choice bioassays revealed that there were significant differences in female preference for certain plants and their volatile oils when compared to the control. Plants of hybrids 3211, 33R87, 33A14, and Dias received more eggs respectively than the control plants. Fewer eggs received the plants of hybrids 31B13 and Aris, with the fewest deposited on plants derived from hybrid 3283W (Table 1). Paired t-test analysis ($P < 0.005$) of the eggs deposited on the control vs. each of the seven hybrid plants revealed that there was significant difference only when hybrids 3211 and 3283W were compared against control plants ($t = 6.786$, $P < 0.001$ and $t = -6.476$, $P < 0.001$, respectively).

Filter paper sticks treated with volatile oils collected from leaves of the hybrid 33A14, 3211, and Dias received more eggs than the controls ($t = 9.398$, $P < 0.001$; $t = 2.567$, $P = 0.040$; and $t = 3.876$, $P = 0.007$, respectively). Sticks treated with the volatile oils of 3283W and Aris received fewer eggs than the controls (Table 1).

Compounds Present in Volatile Oils. More than 20 compounds were detected, characterized, and quantified on the basis of retention time and mass spectra as major constituents of the volatile oils of leaves. No significant qualitative differences were observed among the chemicals identified, but there were quantitative differences in several (Table 2).

The major compound found in all seven hybrids was 3,7,11,15-tetramethyl-2-hexadecen-1-ol (phytol) ranging from 38.3 to 64.9% of the total quantity (total quantity was calculated as the sum of the arbitrary units of all compounds identified in each volatile oil). Compounds detected in high proportions included (*Z*)-3-hexenol (3.1–8%), nonanal (4.9–14.5%), pentadecanal (1.8–5.8%), neophytadiene (5.5–12.9%), (*Z*)-3-hexenyl acetate (2.5–8.9%), and a compound that is an analogue of 2,4-dihydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3-(4*H*)-one (DIMBOA) (2.4–9.3%).

TABLE 1. OVIPOSITION RESPONSE OF *Sesamia nonagrioides* FEMALES TO 30-D-OLD CORN PLANTS OF SEVEN HYBRIDS AND CORN LEAF VOLATILES AFTER STEAM DISTILLATION AND EVALUATED UNDER LABORATORY CONDITIONS IN TWO-CHOICE BIOASSAYS

	Corn plants[a]		Corn leaf volatiles	
Hybrids	Means ± SE (# of eggs)	Mean OI values	Means ± SE (# of eggs)	Mean OI values
31B13	116.3 ± 27.8	−0.18	128.3 ± 24.2	0.37
Control	168.6 ± 37.6		59.4 ± 26.3	
3211	145.6 ± 15.4***	0.77	157.3 ± 18.9*	0.45
Control	19.3 ± 5.7		59.2 ± 19.6	
3283W	55.3 ± 12.3***	−0.71	49.6 ± 20.4*	−0.59
Control	328.9 ± 37.6		194.4 ± 29.3	
33A14	198.7 ± 27.0	0.31	198.3 ± 18.4***	0.78
Control	104.0 ± 25.3		24.9 ± 9.7	
33R87	222.3 ± 31.2	0.33	155.2 ± 35.7	0.23
Control	110.9 ± 24.2		97.9 ± 34.4	
ARIS	169.3 ± 46.2	−0.15	100.7 ± 28.6	− 0.15
Control	229.6 ± 39.8		136.5 ± 25.9	
DIAS	170.2 ± 17.1	0.29	192.3 ± 40.0**	0.74
Control	93.4 ± 25.2		28.2 ± 10.8	

[a] Asterisks indicate significantly different from controls (Paired t test, $N = 15$).
*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

Multivariate Analysis for GC-MS and OIs Data. The original values of each compound identified in each volatile oil were subjected to PCA in order to reduce dimensionality. The application of PCA resulted in the extraction of six principal components (PCs) that accounted for 100% of the total variation of the original data set.

For each principal component, a set of coefficients was calculated that specified how each component is associated with the compounds detected. The set of coefficients is presented in Table 3. A high positive or negative value for the coefficient of a certain compound indicates that this compound has a strong influence on the particular principal component. The first four explained 85.3% of the variance among the hybrids, while PC3 alone explained 34.8%.

If there is a component that best describes the oviposition preference of *S. nonagrioides*, then this should exhibit a significant correlation with the OIs for plants and/or volatile oils. Pearson's correlation coefficients were, thus, computed for the two OIs and the first four principal components.

Results showed that both plants' and volatile oils' OIs were positive and correlated to Principal Component 3 (PC3) ($r = 0.887$, $P = 0.008$ and $r = 0.772$, $P = 0.042$, respectively). The correlation coefficients for the other three PCs with

TABLE 2. MAJOR COMPOUNDS (NORMALIZED ARBITRARY UNITS) IDENTIFIED IN THE STEAM DISTILLATION EXTRACT OF CORN PLANT HYBRIDS[a]

Compound	31B13	3211	3283W	33A14	33R87	ARIS	DIAS
Acid							
Octadecenoic acid	140.12	61.62	29.91	11.16	148.24	75.09	12.33
Alcohol							
(*Z*)-3-Hexenol	185.88	132.08	182.02	129.75	220.13	125.49	326.13
Nonadecanol	20.36	20.75	18.34	13.31	25.98	29.63	26.20
Hexadecenol tetramethyl	28.97	51.88	75.85	23.94	43.03	79.41	70.39
Phytol	890.51	2756.02	1048.63	1048.70	1730.12	2025.59	2241.35
Total	1125.72	2960.73	1324.84	1215.7	2019.26	2260.12	2664.07
Aldehyde							
Nonanal	211.42	212.31	378.87	299.50	168.58	369.70	199.55
Decanal	19.76	41.85	32.56	23.98	22.53	24.11	29.11
Tetradecanal	2.79	6.64	3.01	0.79	4.16	4.70	2.30
Pentadecanal	88.54	193.52	122.29	81.19	202.13	143.13	75.28
Heptadecanal	2.39	6.83	5.85	4.91	3.11	5.83	5.59
Total	324.9	461.15	542.58	410.37	400.51	547.47	311.83
A*lkane-alkene*							
Heneicosane	31.16	60.41	35.49	35.22	41.74	24.31	40.36
Neophytadiene	224.00	286.38	244.41	349.01	215.73	203.72	222.82
Neophytadiene (isomer)	9.56	35.63	11.54	24.49	17.56	25.87	28.37
Total	264.72	382.42	291.44	408.72	275.03	253.9	291.55
Ester							
(*Z*)-3-Hexenyl acetate	149.19	104.97	134.87	242.65	197.89	182.10	343.58
Hydroxyphenyl propenoate	29.74	20.32	0	50.59	24.64	4.69	10.56
Benzyl benzoate	0	10.06	0	0	0	1.41	0
Methyl eicosatrienoate	14.94	50.12	22.39	28.48	51.41	22.05	12.14
Total	193.87	185.47	157.26	321.72	273.94	210.25	366.28
Terpenoid							
Farnesal	6.84	8.40	70.02	7.76	14.15	65.42	19.89
Linalool	4.17	0	0	22.18	48.73	19.73	39.27
trans-Caryophyllene	1.84	0	0	3.53	0	2.57	0
Unknown I	20.27	14.11	11.88	22.69	15.07	18.20	17.68
Uknown II	4.47	3.62	2.96	3.94	6.35	0	0
β-Ionone	3.51	5.93	6.12	4.73	5.69	12.70	12.89
δ-Cadinol	5.84	11.85	8.29	5.28	12.47	34.15	22.62
Uknown III	0	0	0	1.41	0	5.88	3.05
Hexahydrofarnesyl Acetone	19.71	22.16	18.16	21.13	44.69	27.57	25.16
Total	66.65	66.07	117.43	92.65	147.15	186.22	140.56
Other							
DIMBOA analogue	209.42	130.36	145.83	253.23	205.72	86.83	283.95

[a]Three replicates were carried out and the variation was 15–20%.

TABLE 3. EIGENVALUES, PROPORTION OF TOTAL VARIATION DESCRIBED, AND EIGENVECTORS FOR ALL HYBRID VOLATILES IN THE SIX PRINCIPAL COMPONENTS (PC1–PC6)

Principal components	PC1	PC2	PC3	PC4	PC5	PC6
Eigenvalue	6.061	5.035	11.154	5.065	2.935	1.751
Proportion	0.189	0.157	0.349	0.158	0.092	0.055
Eigenvector						
Acids						
Octadecenoic acid	−0.062	0.002	0.263	−0.918	0.215	−0.196
Alcohol						
Z)-3-Hexenol	0.264	−0.457	0.310	0.027	−0.752	−0.245
Nonadecanol	0.880	−0.233	0.185	−0.349	0.078	−0.092
Hexadecenol-tetramethyl	0.850	0.049	−0.457	0.010	−0.229	0.071
Phytol	0.719	0.422	0.473	0.255	0.064	−0.113
Total	0.752	0.362	0.473	0.249	−0.018	−0.131
Aldehyde						
Nonanal	−0.385	0.187	−0.657	0.299	−0.177	0.259
Decanal	0.261	0.827	−0.031	0.424	−0.242	−0.091
Tetradecanal	0.540	0.724	−0.142	−0.296	0.232	−0.152
Pentadecanal	0.329	0.671	0.306	−0.494	0.111	0.303
Heptadecanal	0.482	0.486	−0.232	0.674	0.000	0.153
Total	−0.201	0.709	−0.727	0.011	−0.101	0.429
Alkane–alkene						
Heneicosane	−0.004	0.773	0.520	0.199	−0.278	−0.121
Neophytadiene	−0.645	0.294	0.116	0.620	0.160	0.273
Neophytadiene (isomer)	0.438	0.341	0.470	0.635	0.257	0.039
Total	−0.480	0.446	0.269	0.661	0.124	0.215
Ester						
(*Z*)-3-Hexenyl acetate	0.172	−0.729	0.370	0.453	−0.297	0.095
Hydroxyphenyl propenoate	−0.781	−0.122	0.491	0.152	0.317	0.100
Benzyl benzoate	0.191	0.861	0.239	0.216	0.247	−0.240
Methyl eicosatrienoate	−0.023	0.686	0.522	−0.280	0.086	0.413
Total	0.008	−0.593	0.606	0.451	−0.204	0.192
Terpenoid						
Farnesal	0.503	−0.032	−0.804	−0.081	−0.031	0.304
Linalool	0.265	−0.546	0.640	−0.113	−0.209	0.408
trans-Caryophyllene	−0.374	−0.429	−0.161	0.235	0.756	0.155
Unknown I	−0.416	−0.628	0.222	0.292	0.532	−0.118
Uknown II	−0.669	0.306	0.331	−0.553	−0.085	0.188
β-Ionone	0.873	−0.360	−0.031	0.328	0.011	0.014
δ-Cadinol	0.909	−0.275	−0.031	0.087	0.295	0.043
Unknown III	0.663	−0.483	−0.140	0.297	0.456	0.104
Hexahydrofarnesyl acetone	0.308	−0.131	0.607	−0.558	−0.052	0.454

TABLE 3. CONTINUED

Principal components	PC1	PC2	PC3	PC4	PC5	PC6
Total	0.731	−0.453	−0.088	−0.145	0.038	0.480
Other						
DIMBOA analogue	−0.421	−0.535	0.478	0.294	−0.462	−0.089

the OIs of plants and volatile oils were either negative or not significant [$r = -0.28$ (PC1); $r = -0.22$ (PC2); $r = 0.042$ (PC4) at $P = 0.05$].

The PC3 axis separates hybrids into two distinct groups. The first group (negative OI values) includes plants of hybrids 3283W, Aris, and 31B13. The second group (positive OI values) includes hybrids 33A14, DIAS, 33R87, and 3211 (Figure 1a). For volatile oils, the first group (negative) includes hybrids, 3283W, and Aris, and the second group (positive) includes hybrids 33A14, DIAS, 33R87, and 3211 (Figure 1b). The negative component, where hybrid 3283W and Aris exhibit oviposition deterrence in both plants and volatile oils bioassays, is mainly built by farnesal, nonanal, hexadecenol_tetramethyl, *trans*-caryophyllene, and the sum of the aldehydes, all with high negative coefficients (Table 3). Therefore, it seems that, in terms of chemicals present, the above-mentioned compounds can play a significant role in oviposition deterrence of *S. nonagrioides*.

Oviposition Preference–Synthetic Aliphatic Aldehydes. Results of PCA suggest that the aldehydes and primarily nonanal may play a significant role in the oviposition deterrence of *S. nonagrioides* females. To evaluate this, five standards of C_8–C_{15} straight chain saturated aliphatic aldehydes, most of them found in the volatile oils, were tested. Females deposited fewer eggs on sticks treated with nonanal ($t = -3.946$, $P < 0.001$), decanal, and tetradecanal ($t = 2.574$ and $t = 2.600$, respectively, $P < 0.05$) than those treated with solvent (control). Sticks treated with octanal and pentadecanal received either fewer or greater numbers of eggs than the control, but this difference was not statistically significant (Table 4).

DISCUSSION

Several corn hybrids have been found to possess good levels of resistance to *S. nonagrioides* in field trials (Malvar et al. 1993; Cartea et al., 1999). Our studies suggest that certain hybrids deter females from ovipositing as a result of chemicals produced by these hybrids. The oviposition results obtained were consistent with those reported in a previous paper (Konstantopoulou et al., 2002). Both studies concluded that plants and volatile oils derived from the hybrids 3283W and ARIS deter females from ovipositing.

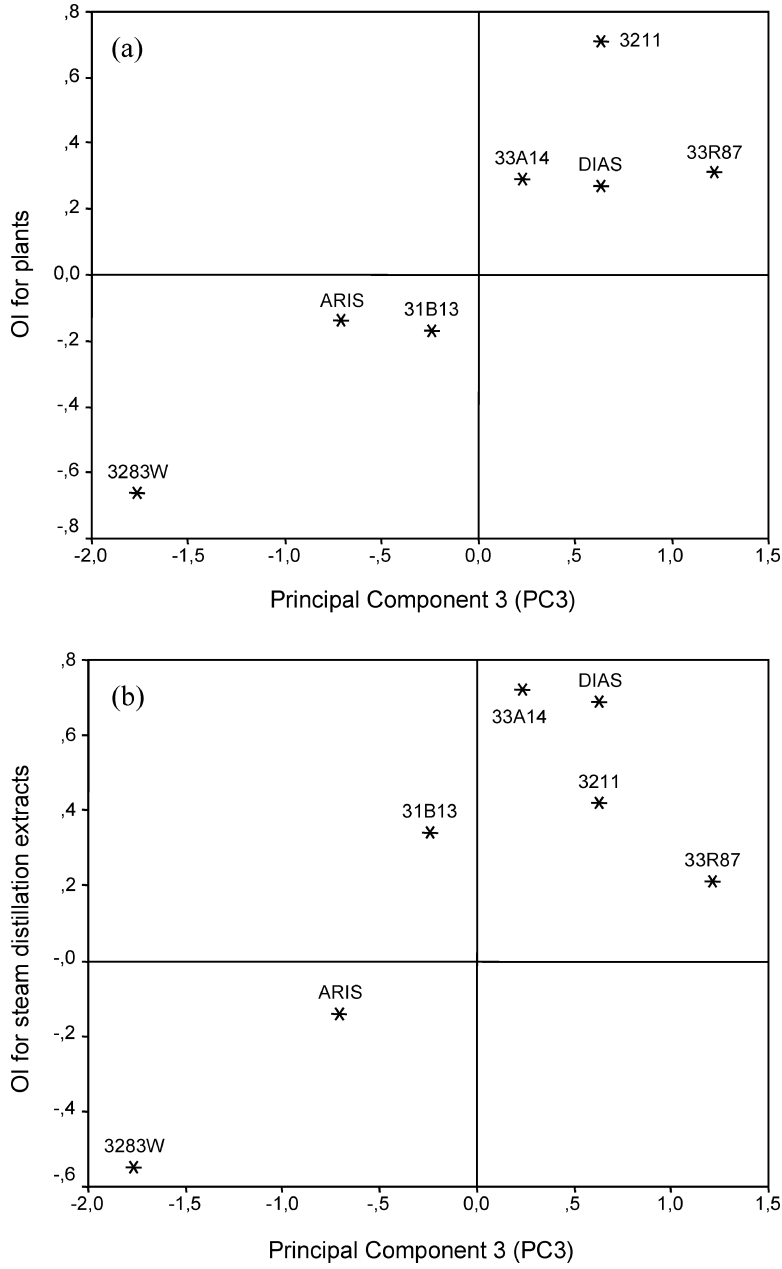

FIG. 1. Scatterplot of (a) OI for plants and PC3 and (b) OI for steam distillation extracts and PC3.

TABLE 4. OVIPOSITION RESPONSE OF *Sesamia nonagrioides* FEMALES TO SYNTHETIC STANDARDS OF SATURATED ALIPHATIC ALDEHYDES EVALUATED UNDER LABORATORY CONDITIONS IN TWO-CHOICE BIOASSAYS (0.5MG/500ML HEXANE)

Synthetic aldehydes	Means ± SE[a] (no. of eggs)	Mean OI values
n-Octanal	79.4 ± 25.2	−0.20
Control	111.9 ± 22.1	
n-Nonanal	4.47 ± 4.47	
Control	123.4 ± 29.8***	−0.93
n-Decanal	62.5 ± 16.6	
Control	298.1 ± 37.8*	−0.65
n-Tetradecanal	112.2 ± 18.0	
Control	256.4 ± 26.0*	−0.38
n-Pentadecanal	161.1 ± 28.0	
Control	77.1 ± 24.4	0.35

[a] Asterisks indicate significantly different from controls (Paired *t*-test, $N = 10$). $*P \leq 0.05$; $***P \leq 0.001$.

GC-MS analyses of the steam-distilled volatile oils resulted in the identification of several major compounds that are similar to the composition of the volatile oils collected from corn tassels and husks, reported by Buttery et al. (1978, 1980). In another study, the major compounds found in corn included the common six-carbon green leaf components, i.e., (*Z*)-3-hexenyl acetate and (*Z*)-3-hexenol, followed by a number of sesquiterpenes (Buttery and Ling, 1984). In our study, (*Z*)-3-hexenyl acetate and (*Z*)-3-hexenol were also found in high proportion, while the proportion of terpenoids detected ranged from 1.6 to 5.1% in the different hybrids examined. The compounds were present in all of the volatile oils analyzed with the exception of the terpenoids linalool and *trans*-caryophyllene. These two compounds were not detected in volatile oils of hybrids 3211 and 3283W. Linalool and *trans*-caryophyllene have been detected in induced corn volatiles emitted from leaves infested by caterpillars (Turlings et al., 2000; Gouinguené et al., 2003). Differences in the relative amounts of compounds detected among the seven hybrids could be attributed to the different genotypes. Gouinguené et al. (2001) reported difference in the relative amounts of induced corn volatiles when several corn genotypes and their wild ancestors were studied.

Steam distillation, due to its harsh conditions, has been suspected as a procedure that can affect the chemicals present in leaves, producing some artifacts. In our case, oviposition preference tests with the volatile oils suggests that the chemicals involved in eliciting oviposition behavior were not affected by the distillation procedure.

Chemometric methods have been applied to GC-MS and ^{1}H-NMR spectra to detect the variability in chemical profiles of fruit juices, olive oils, and

wines, relative to variety, cultivars, and geographical origin (Belton et al., 1998; Mannina et al., 2001; Brescia et al., 2002; Zumin et al., 2004). Such methods applied in our GC-MS and bioassays data revealed compounds with high negative values i.e., farnesal, nonanal, decanal, and tetradecenal that play a key role in the oviposition deterrence of *S. nonagrioides*. Bioassays with synthetic samples showed that C_9–C_{14} aldehydes deter *S. nonagrioides* females from ovipositing, while pentadecanal with a positive value stimulates females. Oviposition deterrence due to the concomitant presence of an aldehyde group and a long straight chain has been previously reported for the European corn borer *Ostrinia nubilalis* (Binder and Robbins, 1997; Binder, 1999). Although compounds with positive PC3 loading were not tested, it is suspected that positive loading compounds such as alkanes and alkenes (heneicosane, neophytadiene) stimulate females to oviposit. Udayagiri and Manson (1997) reported that *n*-alkanes, raging from C_{20} to C_{37} present on corn leaf surface were involved in eliciting oviposition in *O. nubilalis*.

Behavior studies with synthetic aldehydes and the other chemicals with positive PC3 loading are needed to elucidate the role of individual components or their blend on the oviposition behavior of *S. nonagrioides*, and to test the accuracy of the chemometric methods applied. Corn genotypes producing high quantities of C_9–C_{14} aldehydes that deter females from ovipositing could be used as pest management tools for development of effective and environmentally safe control methods for *S. nonagrioides*.

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DEFENSIVE EFFECT OF SURFACE FLAVONOID AGLYCONES OF *Betula pubescens* LEAVES AGAINST FIRST INSTAR *Epirrita autumnata* LARVAE

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Abstract—The surface of birch leaves contains glandular trichomes that secrete exudates containing flavonoid aglycones. We investigated the biological activities of white birch (*Betula pubescens*) leaf surface exudates against larvae of the autumnal moth, *Epirrita autumnata*, a common insect pest of birch. We found that tree-specific mortality (up to 100%) of first instar larvae correlated strongly with the tree-specific contents of surface flavonoid aglycones ($r_s = 0.905$) in emerging leaves. We also found that first instars clearly preferred birch buds from which surface exudates had been removed. In addition, the duration of the first instar was shortened by 29%, and the weights and relative growth rates of first instars improved by 8% and 52%, respectively, as a result of removal of the exudates from their leaf diet. The correlation of tree-specific foliar contents of flavonoid aglycones, especially 5-hydroxy-4′,7-dimethoxyflavanone, with changes in larval performance, suggests that flavonoid aglycones are responsible for the changes observed in first instar larval performance. The results show that chemical characteristics of birch leaves are effective against neonate *E. autumnata* larvae. However, the removal of leaf surface exudates from fully expanded leaves did not affect the leaf acceptance for the voracious fifth instars. This is probably a result of reduction in contents of flavonoid aglycones compared to those of emerging leaves.

Key Words—Flavonoid aglycones, biological activity, birch, *Betula pubescens*, *Epirrita autumnata*, first instar.

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INTRODUCTION

The leaf surfaces of many plants are covered by various nonglandular and glandular trichomes. Together with their lipophilic exudates (flavonoid aglycones, waxes, terpenes, lipids), trichomes may protect leaves against extensive light, UV-B radiation, and desiccation (Ehleringer, 1982; Karabourniotis et al., 1993; Cockell and Knowland, 1999; Tattini et al., 2000; Juma et al., 2001), or they may form the first line of defense against herbivores by entrapping, deterring, or poisoning (Harborne, 1991; Wagner, 1991; Hare, 2002; Zalucki et al., 2002). For Solanaceae, in particular, trichome exudates are responsible for the mortality of neonate larvae; the removal of exudates using an ethanol solution increase larval survival (Gurr and McGrath, 2002). In addition, the removal of exudates from the leaf surface increases the consumption rates of neonates (van Dam and Hare, 1998) and their mobility on the leaf surface, shortens the duration of larval development, and even increases pupal weight (Malakar and Tingey, 2000). In general, the highest mortality of lepidopteran larvae occurs during the first instar (Zalucki et al., 2002); such an effect in the first stages of larval development is the most beneficial, since it can dramatically reduce future biomass losses of the plant (Karban and Baldwin, 1997).

Earlier studies on the mechanisms of birch resistance to herbivores have focused primarily on the role of internal leaf phenolics against larvae of the autumnal moth *E. autumnata* (Kause et al., 1999a; Ossipov et al., 2001; Haukioja et al., 2002; Henriksson et al., 2003). In a recent study (Valkama et al., 2003), we discovered that the leaf surfaces of four Finnish birch species contain flavonoid aglycones, which are secreted by glandular trichomes. In all the species studied, these epicuticular flavonoids peaked in young leaves, with white birch (*Betula pubescens* ssp. *pubescens*) containing the highest levels, up to 10% of leaf dry weight (Valkama et al., 2003). In this study, we investigated the biological activities of birch leaf glandular trichome exudates during the first developmental phase of *E. autumnata* larvae. One of our aims was to determine whether the performance of first instars differed when fed with exudate-free or exudate-containing leaf diets. A specific goal was to determine the component of the exudate with the strongest effect on first instars and to compare its efficiency with regard to fifth, i.e., last, instars.

METHODS AND MATERIALS

Study Organisms. The autumnal moth [*Epirrita autumnata* (Borkhausen)] is a polyphagous geometrid moth commonly found on white birch (*Betula pubescens* ssp. *pubescens*) and other birch species in northern Europe. It is a destructive pest, especially in northern and high-altitude populations of mountain birch [*Betula pubescens* ssp. *czerepanovii* (Orlova) Hämet-Ahti] (Tenow, 1972; Haukioja et al., 1988; Ruohomäki et al., 2000). The larvae hatch in the spring, simultaneously

with bud break, and feed on the young foliage. It is important for the larvae to reach pupation before the rapid decline in the quality of maturing birch leaves (Kause et al., 1999b, Riipi et al., 2002; Haukioja, 2003).

Pilot Bioassay. We reared larvae of *E. autumnata* on the leaves of eight individual white birch trees. The experiment was conducted in 2001, at the Botanical Garden of Turku University in Turku, Finland. The phenological stage of each tree was classified at the beginning of the experiment (May 8) based on the amount of green leaf material visible from the bud. Twenty-eight larvae were reared in 48-ml plastic vials with buds/emerging leaves from each study tree. At the beginning of the experiment, the neonates were offered a single bud; to prevent leaf wilting, the buds/leaves were changed every other day. Vials were kept at 20°C throughout the experiment. We recorded the mortality of larvae for the whole first instar. We collected leaf samples for the analysis of foliar flavonoid content on May 14. Since the experiments were initiated on the same day for all the trees, we were able to study tree-specific dependence of larval mortality on tree phenology and the contents of leaf surface flavonoids.

Cafeteria Experiment with First Instar Epirrita autumnata. In a 24-hr cafeteria experiment, first instar *E. autumnata* were allowed to choose among control buds and buds from which sticky exudates had been washed away. Ten individual white birch (*Betula pubescens* Ehrh.) trees growing in the Botanical garden of the University of Turku were chosen, and 24 buds (attached to 5- to 10-cm-long twigs) were collected from each tree. Timing of the cafeteria experiment was April 29–May 7, 2002; bud collection was conducted for each tree, based on the tree-specific phenology, and the phenological stages of the buds were similar for different trees at the time of the experiment.

Twelve of the 24 buds were washed by immersing them in 50 ml of 95% ethanol for 10 sec and subsequently in 50 ml of water for 5 sec to remove traces of ethanol. Excess water was gently removed by placing the leaf between pieces of filter paper. This procedure removed the lipophilic substances—including the flavonoid aglycones—dissolved in the sticky exudates, as shown by the lack of stickiness in the washed buds compared to unwashed control ones, and by the appearance of flavonoid aglycones (detected by HPLC) in the yellow-colored ethanol solution. The other half of the buds was immersed for 5 sec in 50 ml of water only, and the excess water was removed as above; these control buds still contained the lipophilic surface components. The two bud diets were offered to each larva on a Petri dish. First instars of *E. autumnata* ($N = 120$), from four different broods of southern Finnish origin, were used in the experiment; each larva received diets originating from a single tree. After 24 hr, we observed which bud the larvae had visited (visible holes in buds or drops of feces beside the bud), and calculated the percentage of buds eaten to determine which diet was preferred.

No-Choice Experiment with First Instar Epirrita autumnata. To test the effects of removal of trichome exudates from the leaf diet on performance of first instars over a longer time-scale, we carried out a bioassay lasting throughout the first

instar (29 April–14 May, 2002). Larvae ($N = 128$), from four different broods of southern Finnish origin, were fed buds from eight trees. Half received buds from which the surface exudates were washed away, and the other half unwashed control buds (similar diets as described above). One larva and one bud were placed in a 20-ml vial, which was kept under a regime of ambient temperature (daily variation approx. 4°C–26°C) and light (approx. 16:8 L:D). Buds were replaced with fresh ones every day until the larvae molted into the second instar. The duration of the first instar and the weight of each larva were measured at the end of the experiment. The relative growth rate of each larva was calculated by dividing $\log_e$ (final weight of the first instar larva) by the duration of the first instar (in days).

No-Choice Experiment with Fifth Instar Epirrita autumnata. A 48-hr bioassay was conducted to study the effects of the presence/absence of leaf surface exudates, and especially 5-hydroxy-4′,7-dimethoxyflavanone (which was found most effective against first instars) on the performance of the fifth instars (June 2–4, 2003). We fed larvae with control leaves, leaves from which surface exudates had been washed away, and leaves which had been washed and painted with 5-hydroxy-4′,7-dimethoxyflavanone at a level of 10 mg/g leaf dry weight (for practical reasons this is not possible to do with buds or very young, wrinkled leaves consumed by first instars). This level was chosen because it is higher than the average content of this compound (identified as Flavanone in Valkama et al., 2003) at any stage of the growing season (contents vary from 4.2 to 1.4 mg/g during the leaf development, Valkama et al., 2004). The painted compound was dissolved in 90% acetone, and known volumes were painted onto the surface of fresh birch leaves (originating from a single white birch growing in the Botanical Garden) to approximate the desired content. The washed controls were painted with 90% acetone only. Details of the painting procedure have been described by Salminen and Lempa (2002). The only exception to the method was that before painting the leaves were washed with 95% ethanol, as in the cafeteria experiment with the first instars, to remove the flavonoids on the birch leaf surface. We used 24 larvae of *E. autumnata*, from four different broods of southern Finnish origin. Eight larvae were randomly allocated to the leaves of each treatment. Larvae and leaves were placed in 20-ml vials at 15°C and approx. 16:8 L:D. The bioassay was a no-choice experiment: larvae were placed individually in vials and received only one type of food. Leaves were replaced after 24 hr. The initial and final weights of the larvae were measured for the calculation of growth during the experiment.

Bud and Leaf Sample Preparation. Samples (buds or leaves) were collected at the beginning of each bioassay to monitor the foliar phenolic composition of the experimental trees. To analyze the lipophilic phenolics, freeze-dried buds and leaves were extracted $\times 3$ for 1 hr, with 95% ethanol (3×3 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 Analyses. Extracts were analyzed with HPLC-DAD at 280 and 349 nm. The HPLC system (Merck-Hitachi, Tokyo, Japan) consisted of a pump L-7100, a diode array detector L-7455, a programmable autosampler L-7250, and an interface D-7000. Column and chromatographic conditions were as described earlier (Salminen et al., 1999), except that 0.1 M H_3PO_4 was replaced with 0.05 M H_3PO_4. Lipophilic flavonoid aglycones were identified on the basis of their UV and mass spectra and retention times as reported in the literature (Valkama et al., 2003), and quantified (absolute amounts) as acacetins (flavonols and flavones) or naringenins (flavanones).

GC-MS Analyses. The leaf extracts of the no-choice experiment with the first instars were also analyzed with the GC-MS. For the analysis of lipids and fatty acids, 4 ml of ethanol extract of each tree were evaporated under nitrogen flow to dryness. Dry residues were dissolved with 3 ml of chloroform/methanol (2/3), and 50 μl of internal standard (nonadecanoic acid methyl ester, 2 mg/ml in chloroform solution) to which 60 μl of sulfuric acid were added. After methylation of fatty acids and transmethylation of lipids (100°C, 4 hr), samples were cooled, and sulfuric acid was extracted $\times 4$ with 4 ml of water. The chloroform fraction was evaporated under nitrogen flow to dryness, the residue was dissolved to 100 μl of mixture chloroform/pyridine (1/1, v/v), and lipophilic compounds, other than fatty acids, were trimethylsilylated by adding 100 μl of *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA, Sigma).

Because of the possibility that the methylation procedure could cause the loss of some phenolics, terpenoids, or steroids, extracts were also analyzed directly trimethylsilylated. For these GC-MS analyses, a 2-ml sample of ethanol extract of each tree with 25μl of internal standard (nonadecanoic acid methyl ester, 2 mg/ml) was vacuum dried and trimethylsilylated. Dry residues were dissolved in 50μl of mixture pyridine/chloroform (1/1, v/v) and 50μl MSTFA with 5% trimethylchlorosilane as a catalyst (Sigma-Aldrich) and incubated for 1 hr at 50°C and 24 hr at room temperature.

Derivatives of lipophilic compounds were analyzed with the Perkin-Elmer Autosystem XL/TurboMass Gold GC-MS system (Norwalk, CT, USA). MS was used in the electron impact mode (EI^+); the data acquisition scan time was 0.45 sec and the scan range from 50 to 620 *m*/*z*. The column was a Perkin-Elmer capillary column (PE-5MS, 30 m, 0.25 mm i.d., film 0.25 μm), and helium was used as a carrier gas with a flow rate 1.0 ml/min. The injection volume was 1.0 μl, and the split ratio was 20:1 for fatty acids, and 2.0 μl and 15:1 for trimethylsilylated samples. For fatty acids, the initial temperature of the injector was 280°C. The oven temperature was programmed as follows: initial temperature 75°C (2.0 min) followed by a temperature increase of 5°C/min up to 250°C; this was held for 25.0 min, then 15°C/min to final temperature 290°C, held for 15.0 min. Total run time was 80 min. For the trimethylsilylated samples, the initial temperature of

the injector was 300°C, and the inlet line and the MS source were held at 310 and 200°C, the correspondingly. Oven temperature was programmed as follows: initial temperature 110°C (2.0 min) followed by temperature increase of 4°C/min up to 260°C, and then 1.5°C/min to final temperature 310°C, which was held for 17.17 min. Total run time was 90 min. Compounds were quantified relatively (peak area/g dryness) by normalizing the intensity of individual ion traces to the response of the internal standards and to 1 g of plant leaf dry weight. Correlations with the absolute amounts obtained from the HPLC-DAD method were high.

Isolation and Identification of 5-Hydroxy-4′,7-Dimethoxyflavanone. To isolate a previously unknown white birch leaf flavanone with a high-positive correlation with the mortality of first instar *E. autumnata* larvae, for structural elucidation, a large amount of freeze-dried white birch buds (10 g) was extracted with 95% ethanol. The evaporated extract was dissolved in petroleum ether and fractionated on a silica column (10 × 2.0 cm i.d., 70–230 mesh, 60 Å) with 50-ml fractions of petroleum ether containing increasing amounts of ethyl acetate. On the basis of HPLC-DAD analysis, the flavanone was detected in the second fraction, which was eluted with 20% ethyl acetate in petroleum ether. The fraction was further purified with a Merck LiChroprep preparative RP-18 column (44 × 3.7 cm i.d., 40–63 μm) using a step gradient of CH_3CN (from 5 to 70%) in H_2O to yield the pure compound.

NMR-spectra were acquired using a Bruker Avance 500 spectrometer (equipped with a BBI-5mm-Zgrad-ATM probe) operating at 500.13 MHz for ^{1}H and 125.77 MHz for ^{13}C. Spectra were recorded at 25°C using acetone-d_6 as a solvent. Proton and carbon spectra were referenced internally to the TMS signal using a value 0.00 ppm. In addition to standard proton and carbon spectra, the 2D gradient selected DQF-COSY, HSQC, and HMBC spectra were also measured.

Statistical Analyses. We analyzed the preference of first instars in the cafeteria experiment with χ^2 test (proc FREQ, SAS Inc., 1996). The growth parameters of the first and fifth instars were analyzed with analysis of variance (Proc GLM, SAS Inc., 1996). In the analysis of the fifth instars, the change in larval weight during the experiment (growth) was used as the dependent variable, with initial larval weight as covariate. The assumptions of analysis of variance, normal distribution of residuals, and homoscedacity of treatment variances were tested with Kolmogorov-Smirnov test and Levene's test, respectively.

RESULTS AND DISCUSSION

Pilot Bioassay. In a preliminary bioassay, 28 neonate larvae per tree were reared on young white birch leaves, and mortality during the first instar was recorded. Tree-specific mortality during the first instar displayed large among-tree variation (from 36 to 100%), being highest in trees with the least

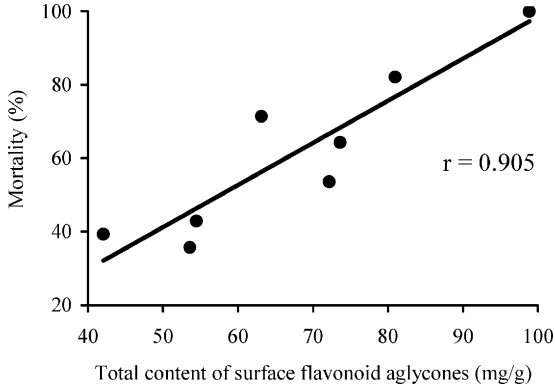

FIG. 1. Mortality of first instar *Epirrita autumnata* larvae relative to the total content of surface flavonoids.

advanced phenology, i.e., with the youngest leaves ($r_s = 0.901$). In mountain birch, young leaves are nutritionally superior to older ones because of declining concentrations of water and amino acids (amino acid contents decrease from buds to senescing leaves from 200 to 44 mg/g) (Riipi et al., 2002). However, our results suggest that in the case of opening buds, leaf defenses peaking in young leaves override the possible differences between trees in the nutritive value of emerging leaves. Interestingly, tree-specific larval mortality correlated strongly positively ($r_s = 0.905$, $P < 0.002$) (Figure 1) with the content of tree-specific levels of flavonoid aglycones. Because of the strong correlation between flavonoid aglycone content, larval mortality, and tree phenology (data not shown), we attempted to establish a causal link with a more detailed experimental approach.

Effects of Leaf Surface Exudates on the Performance of First Instar Epirrita autumnata. In the cafeteria experiment, 120 larvae of first instars were allowed to choose for 24 hr between control buds and buds from which the surface exudates had been washed away. After that time, 63% of the larvae were found eating the washed bud (Figure 2a); none of these larvae left visual evidence (a hole in the bud or feces beside the bud) of biting the control bud. While only 23% of the insects were found eating the unwashed control bud, 13% did not choose either diet. These findings suggest that *E. autumnata* larvae prefer buds from which the lipophilic exudates have been removed.

In the no-choice experiment lasting throughout the first instar, we found that the duration of the instar was shortened by 29% following removal of exudates from the diet (Figure 2b). This shortening of instar duration indicates more rapid growth for larvae fed with exudate-free buds than for larvae fed with control buds. The superiority of washed leaves over unwashed ones also in terms of weight and relative growth rates (RGR) of first instars demonstrated the significance

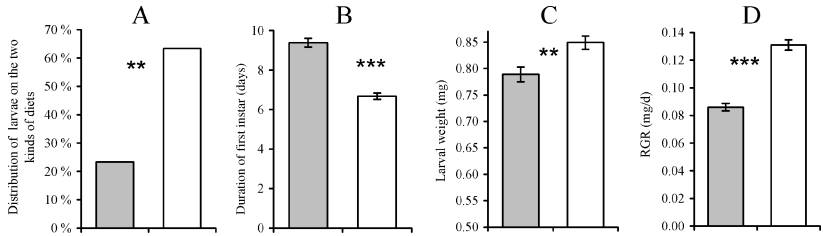

FIG. 2. Differences in the performance of first instar *Epirrita autumnata* larvae feeding either on a control (unwashed leaves) diet (grey bars) or on a diet from which surface exudates had been washed away with 95% ethanol (white bars). (A) Distribution of larvae on control and washed buds in the cafeteria experiment after 24 hr, $N = 120$ [16 larvae (13%) did not choose either of the diets]; (B) duration of the first instar; (C) larval weights in the end of the first instar; (D) relative growth rates during the first instar on different diets, N=128. Statistically significant differences: $^{**}P < 0.01$; $^{***}P < 0.001$.

of exudate removal (Figure 2c–d). The weights and relative growth rates of the first instars improved by 8 and 52%, respectively, as a result of the removal of the exudates. Furthermore, since immersing the leaves in ethanol resulted in the removal of exudates but not of glandular or nonglandular trichomes (E. Valkama, unpublished scanning electron microscopy data), it is evident that the exudates, not the trichomes *per se*, were specifically responsible for the negative effect on the performance of *E. autumnata* consuming the control diet.

Finding the Active Component of the Exudate Against First Instar Epirrita autumnata. Since birch leaf trichomes may secrete other types of compounds besides flavonoids, for example, essential oils (Inki and Väisänen, 1980; Isidorov et al., 2004), the potential causal role of flavonoid aglycones was examined in more detail. We, therefore, correlated changes in the mean RGR and the mean duration of the first instar from tree to tree due to the presence/absence of surface exudates, to determine whether the magnitude of these changes correlates with tree-specific levels of flavonoid aglycones or other lipophilic substances detected with HPLC-DAD or GC-MS.

Flavonoid aglycones seemed to represent the factor that decreased the development rate of first instars, because we found that the richer in flavonoid aglycones the leaves were, the more washing them away improved larval performance (Figure 3). The content of total flavonoid aglycones in leaves, which were fed to first instars, varied between 52 and 101 mg/g. To identify the most active flavonoid aglycone among the 18 individual compounds studied (15 of which were the same compounds as reported in Valkama et al., 2003), their contents, analyzed with HPLC-DAD, were separately correlated with RGR and instar duration. The r values varied between 0.15 and 0.85 with RGR and between -0.07 and -0.93 with instar duration. In both cases, the strongest correlations were related to the same

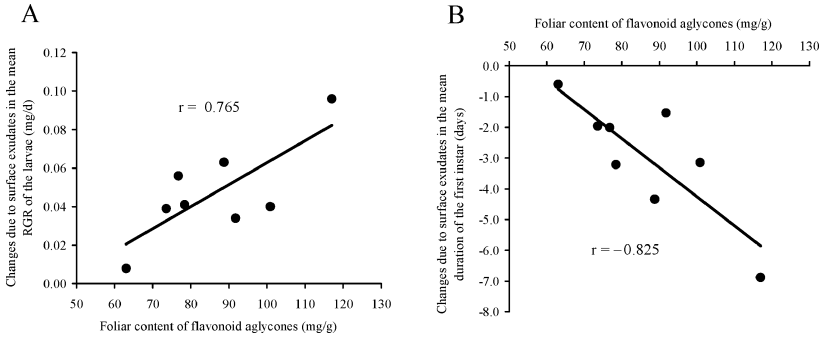

FIG. 3. Increase in larval growth rate (A, $N = 8$) and decrease in duration of first instar (B, $N = 8$) caused by washing away leaf surface exudates. Values are tree-specific means ($N = 8$) and $P < 0.05$ for both correlations. The *y*-axis values were calculated by subtracting the mean RGR or instar duration values for larvae consuming control leaves from the mean values for larvae consuming washed leaves of the same trees.

aglycone. This compound was isolated from the leaf surface exudate, and identified on the basis of NMR results as 5-hydroxy-4′,7-dimethoxyflavanone. The positions of the methoxy groups were unequivocally determined by the HMBC spectrum. The carbon chemical shifts agreed well with those reported by Rossi et al. (1997).

To study the possible defensive activity of surface compounds other than flavonoid aglycones, similar correlations between larval performance and concentrations of lipophilic compounds detected by GC-MS were computed. Only weak correlation values were found for concentrations of individual fatty acids or for their total content (for individual compounds the *r* values for concentration vs. RGR varied between 0.01 and 0.20, and for concentration vs. duration of the first instar between −0.02 and −0.10). In the samples, which were only trimethylsilylated, we found 140 compound peaks that belong to groups of terpenoids, steroids, hydrocarbons, fatty acids, and flavonoid aglycones (Ossipov, unpublished observations). However, strong correlations were found again between concentrations of flavonoid aglycones and RGR or instar duration as in the HPLC-measurements, in addition to that between RGR and the contents of two long-chain hydrocarbons.

Effects of Leaf Surface Exudates on the Performance of Fifth Instar Epirrita autumnata. As in the first instar experiments, the fifth (i.e., last) instars were also fed with control leaves and leaves from which the exudates had been washed away. However, when larvae are in their fifth instar, leaves are almost full-grown, and, therefore, have greatly reduced contents of trichome-exuded flavonoid aglycones (average value 20 mg/g dw) compared to those of newly flushed leaves (average value 70 mg/g) (Valkama et al., in press), on which neonate larvae fed in the

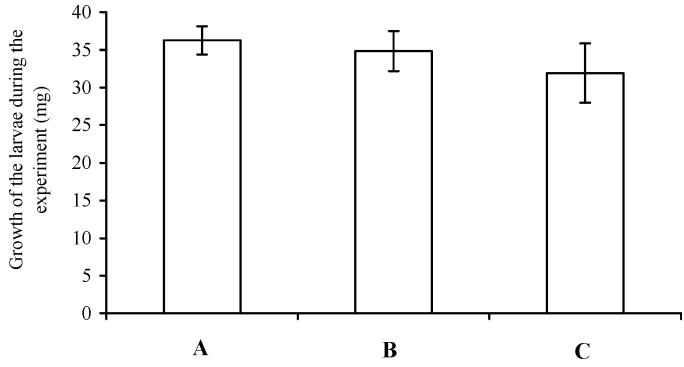

FIG. 4. Growth of fifth instar *Epirrita autumnata* larvae feeding either on unwashed control leaves (A), 95% ethanol washed leaves (B), or 95% ethanol washed and 5-hydroxy-4′,7-dimethoxyflavanone painted leaves (C). Bars show mean ± SE; $N = 8, 7$, and 7 in different treatments.

no-choice experiment. Accordingly, we found no significant differences in the performance of fifth instars between the two diets (Figure 4). Fifth instars were also fed leaves with artificially enriched levels (10 mg/g dw) of 5-hydroxy-4′,7-dimethoxyflavanone to study the possible toxic effects of this compound, which was the most defensive against first instars. Despite the effects on growth and instar duration of neonates, fifth instars did not grow significantly more poorly than larvae fed on control or washed leaves. These findings suggest that surface flavonoid aglycones in fully expanded birch leaves do not form a barrier against fifth instars, and this is probably a result of considerable reduction flavonoid aglycones through leaf development.

Ecological Implications of the Observed Effects of Leaf Surface Exudates on Epirrita autumnata. The observed strong effects of surface exudates on the preference and performance of first instar *E. autumnata* on white birch offer a new component for our understanding of birch defense against herbivores. The strong preference of first instars for washed buds suggests that surface exudates have the potential to repel neonates (for other lepidopteran species see van Dam and Hare, 1998). The exudates provide two direct defenses for birch. First, the sticky surface of young leaves limits feeding by neonates; second, exudates reduce the growth rate of first instars. These negative effects on growth and developmental rate in turn may be beneficial to birch for two reasons. First, larval development may not be able to keep pace with the development of a birch leaf. This forces the larvae to consume relatively old and low-quality leaves, which in turn may reduce larval growth and leaf consumption (Haukioja, 2003). Second, prolonged development may make the larvae more vulnerable to parasitoids (Kaitaniemi and Ruohomäki, 1999). Our results imply that these defenses can occur at foliar flavonoid aglycone

levels higher than 60 mg/g (Figure 3). The total content of surface flavonoids on buds or very young white birch leaves varied between 50 and 100 mg/g, with a mean content of 80 mg/g, which clearly exceeds the determined defensive level of these compounds.

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EFFECTS OF PURIFIED PERSIMMON TANNIN AND TANNIC ACID ON SURVIVAL AND REPRODUCTION OF BEAN BUG, *Riptortus clavatus*

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Abstract—We evaluated the effects of tannic acid and purified persimmon tannin on survival and reproduction of bean bugs, *Riptortus clavatus*. Feeding behavior of *R. clavatus* was also examined on sweet (cv. Fuyu) and astringent (cv. Chongdosi) persimmon fruits. Soluble tannin in sweet persimmon fruits decreased from 3% in early June to 0.5% in late September, but it increased from 2 to 8% during the same period in astringent persimmon fruits. More bugs visited sweet than astringent persimmon. Numbers of piercing/sucking spots were higher on sweet than on astringent persimmon. When fed 1 and 3% solutions of persimmon tannin, adult bugs ingested only 64.1 and 9.5% of the amount of water ingested by those offered the control (distilled water). Amounts of persimmon tannin ingested by the adult bugs were 6.5 and 2.8 times higher at 1 and 3% tannin solutions compared to a 0.1% solution. Persimmon tannin exerted negative effects on survival and reproduction of *R. clavatus* at higher concentrations (1 and 3% solutions). Feeding of *R. clavatus* adults decreased with increasing tannin concentrations. When results from both sexes were pooled, 50% mortality was achieved at 11 and 4 days after treatment with the 1 and 3% tannin solutions, respectively. Reproduction decreased with 1% tannin, and no eggs were produced with 3% tannin solution. Tannic acid was similar in its effects on *R. clavatus*. All nymphs died 14, 12, and 7 days after feeding on 0.1, 1, and 3% tannic acid, respectively. Adults were less sensitive than nymphs, and their survival was not affected by 0.1% tannic acid. However, 1 and 3% tannic acid solutions were fatal. Survivorship decreased to 50% at 11

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and 6 days after supplying tannin solutions of 1 and 3% concentrations. Higher concentrations (1 and 3%) resulted in reduced reproduction, as was seen with persimmon tannin. Our data may explain why *R. clavatus* does not invade sweet persimmon orchards until late July, when concentrations of soluble tannin are low enough to allow them to feed.

Key Words—Persimmon, tannins, tannic acid, *Riptortus clavatus*, survival, reproduction.

INTRODUCTION

Many plants, including persimmon (*Diospyros kaki* Thunb.) fruit, contain tannins, plant polyphenols of high molecular weight (Taira, 1996). Persimmon tannins cause an astringent sensation in the mouth. The fruits are strongly astringent when small and immature. Sweet persimmon fruits lose their astringency by the time fruits with firm flesh are harvested. Astringent persimmon fruits, however, remain so and are inedible even when fully colored (Taira, 1996). Tannin is contained in tannin cells in persimmon fruits; the tannin content varies with different varieties and with seasons. Tannin cells in astringent persimmon fruits and in young sweet persimmon fruits are easily damaged, and soluble tannins are released, producing an astringent sensation (Lee and Kim, 1972). Tannin content in Jiro (a variety of sweet persimmon) and Fuyu persimmon range from 3% in young fruit to 0% at harvesting time (Inaba et al., 1971; Taira, 1996).

Plant tannins have received much attention since the early works of Feeny (1968, 1970) suggesting that oak tannin was important for the protection of foliage from caterpillar damage. Plant tannins can serve as antiherbivory defense agents against many generalist herbivores, and their effects are proportional to their levels in the plant (Scriber and Ayres, 1988; Bernays et al., 1989; Schultz, 1989; Clausen et al., 1992; Feeny, 1992; Nomura and Itioka, 2002). However, this generalization has been challenged by some (Bernays et al., 1980, 1981; Smith et al., 1992; McArthur and Sanson, 1993; Panzuto et al., 2002). Bernays et al. (1980) found that tannic acid was actually a feeding stimulant in various species of Acridoidea and that it improved growth of the tree locust, *Anacridium melanorhodon*. Bernays et al. (1981) showed that condensed tannin sprayed on wheat leaves was not deleterious to survival and growth of four species of Acridoidea at levels below about 10% dry weight on food. At higher concentrations, however, consumption and efficiency of conversion of digestion were reduced. Panzuto et al. (2002) concluded that, although *Choristoneura rosaceana* larvae develop faster on a tannic acid diet, this compound is not a phagostimulant, and they suggested that not all phagostimulants are necessarily beneficial to an insect's fitness.

The most important insect pests on persimmon are stink bugs such as the malmorated stink bug (*Halyomorpha halys*), the bean bug (*Riptortus clavatus*), and the green stink bug (*Plautia stali*) (Kawada and Kitamura, 1983; Chung et al., 1995; Adachi, 1998; Lee et al., 2002). Damaged fruit parts turn blackish-green and

become concave, and the fruit are not marketable. Insects invade sweet persimmon orchards and suck fruit juices mainly from late July (Chung et al., 1995; Lee et al., 2002). Damage levels caused by the bugs are negligible before then (Chung et al., 1995). Our observations over a number of years indicated that *R. clavatus*, which overwinter as adults, occurred in abundance from late April in Korea (Park et al., unpublished data). However, the main period of invasion of sweet persimmon orchards is late August (Chung et al., 1995). We assumed that the content of soluble tannins in fruits is responsible for the difference between peak period of damage to sweet fruits and the phenology of the adult bugs. Persimmon contains both hydrolyzable and condensed tannins, and the proportions of the two groups change with growth of the fruit (Matsuo and Itoo, 1981).

In this study, we measured the dosage effects of persimmon tannin on the bean bug that is known to feed on mainly sweet persimmon and on soybean (Chung et al., 1995; Son et al., 2000). Different plant tannins have varying effects on different herbivore species, and the same tannin can have different effects on different herbivores presumably because of interactions between tannin structure and gut physiology (Ayres et al., 1997). For this reason, we also tested the effect of a commercially available tannic acid on the bean bug.

METHODS AND MATERIALS

Insects. Bean bugs, *Riptortus clavatus*, (Hemiptera: Alydidae) used in this study were from a field collected colony maintained in the laboratory for 2 yr. Insects were reared in round plastic containers (15 × 7.5 cm) with netting covers, at 25 ± 1°C and a 16L:8D photoperiod. Larvae and adults were supplied with soybean seeds, peanuts, and distilled water. To prevent decrease of insect vigor, the laboratory colony was supplemented once or twice a year with field-collected bugs.

Chemicals. Tannic acid powder (ACS reagent), HCL, and MeOH were purchased from Sigma-Aldrich Fine Chemicals (Missouri, USA). Persimmon tannin was supplied by Maruzen Pharmaceuticals Co. (Hiroshima, Japan). This tannin was purified from immature astringent persimmons by fermenting the juice with yeast to dissolve and remove pectin. The juice was then absorbed onto a porous adhesive resin column, washed with water, and eluted with alcohol to get high molecular weight tannin. After decoloration and freeze-drying, persimmon tannin of >95% purity was obtained. The structure of the above tannin was determined by thin-layer chromatography, polarimetry, and UV and IR spectrometry. It is composed of catechin, gallocatechin, catechin-gallate, and gallocatechin-gallate (1:0:3:2, w/w) that, by ultrafiltration, was found to be composed of units with molecular weights of <1000 (4.218%), 1000–10,000 (3.87%), and >10,000 (92.06%). Impurities consisted of heavy metals (<50 ppm), arsenic (<2 ppm), and iron (<35 ppm) salts.

Seasonal Changes of Tannin Contents in Sweet and Astringent Persimmon Fruits. Fruits of astringent (cv. Chongdosi) and sweet (cv. Fuyu) persimmon were collected randomly at 10-day intervals from June 10 to September 30, 2001. One fruit from each of five trees per variety was sampled on every sampling date. Soluble and insoluble tannins were measured by the Folin–Denis method (Taira, 1996). Soluble tannins were extracted twice at room temperature by homogenizing 10 g of fruit flesh with 100 ml of 80% MeOH. Insoluble tannin was assayed in the residue of the above extract. The residue was resuspended and extracted under reflux conditions with 1% HCL in MeOH at 80°C. The mixed solution of the extract (5 ml) and Folin–Denis reagent (5 ml) was sonicated, kept for 3 min at room temperature, and then 5 ml of Na_2CO_3 solution were added. After keeping the mixed solution at room temperature for an hour, its absorbance was determined at 760 nm by UV/VIS spectrophotometry (UV-1601, Shimadzu Co., Japan). The amount of tannin was converted to equivalent tannic acid based on the standard curve for tannic acid. Tannin content was expressed as a percentage of tannin weight to fresh weight of fruit flesh.

Feeding Behavior on Astringent and Sweet Persimmon Fruits. Two varieties, Fuyu and Chongdosi, were used. They were purchased from a farmer who harvested the fruits and stored them in a large low temperature chamber (-1.0 ± 0.5°C). Female adults of the bean bug were starved for 24 hr. Twenty females were placed on two astringent and two sweet persimmon fruits separated by 10 cm in a plastic cage ($25 \times 25 \times 25$ cm). Feeding behavior of the bugs was photographed at 1-min intervals for 24 hr with a digital video camera recorder (DCR-PC115, Sony, Japan). The number of bugs visiting the two different kinds of persimmon fruits was counted at hourly intervals by viewing the recorded videotapes. Numbers of visitors were pooled at 3-hr intervals. After 24 hr, those fruits were peeled with a sharp knife to examine the number of piercing/sucking spots in the fruits. This experiment was done at 25 ± 1°C and a 16L:8D photoperiod with three replicates.

Preparation of Tannin Solutions. Samples of tannic acid and persimmon tannins were dissolved in distilled water to give different concentrations. Fresh solutions were prepared each day before use except for the experiment on the effects of tannic acid on survival and fecundity of adults. A glass vial was filled with a tannin solution and plugged with rolled filter paper (Whatman No. 1). A vial containing tannin solution, five soybean seeds, and two peanuts were placed in a round plastic container (15×7.5 cm) that contained test insects.

Effects of Persimmon Tannin on Bean Bugs

Relative Feeding Amount. Powder of persimmon tannin was dissolved in distilled water to give concentrations of 0.1, 1, and 3% (w/v). Tannin solutions were supplied to the bugs as mentioned above. Ten 5–10-day-old adult males were

placed into a round plastic container (15 × 7.5 cm) that contained a vial of tannin solution, and adequate amounts of soybean seeds and peanuts. No water or distilled water was given instead of tannin solution to bugs as control and positive control treatments, respectively. Filter paper was placed onto the bottom of the container to capture the bugs' excreta; the paper was changed each day for 10 days. Areas of the filter paper stained by the secretions were carefully cut out, dried in an oven (60°C) for 3 hr, and weighed. The weight of this filter paper was used to calculate a relative index of the amounts of water ingested by the bugs. Weight values were multiplied by the concentration of the tannin solution of the respective treatment. These calculated values were used as an index of tannin amounts ingested by the bugs. This experiment was done in triplicate.

Effects on Survival and Fecundity of Adults. Concentrations of 0.1, 1, and 3% (w/v) tannin solution were prepared by dissolving persimmon tannin in distilled water. Ten pairs of adult bugs within 24 hr after emergence were placed into a round plastic container that contained a vial of tannin solution with a few soybean seeds and peanuts. In the control treatment, a vial of distilled water was provided instead of tannin solution. This was replicated three times. Numbers of dead insects and eggs oviposited were obtained each day for 33 days, 1 week later than when all bugs in the 0.1, 1.0, and 3.0% treatments had died. Survivorship was expressed as percentage survival by days, and fecundity as the number of eggs per female per day.

Effects of Tannic Acid

Effects on Survival of the Nymphs. Five first-instar larvae within 24 hr after hatching were placed into each of 12 round plastic containers (15 × 7.5 cm). Powder of tannic acid was dissolved in distilled water to give 0.1, 1.0, and 3.0% concentrations (w/v). Distilled water was used as a control. Dried soybean seeds and peanuts were supplied as food. Dead nymphs were counted each day up to 14 days after infestation when all nymphs fed 0.1% tannin solution had died.

Effects on Survival and Fecundity of Adults. Solutions of 0.01, 0.1, 1, and 3% (w/v) tannic acid powder were prepared by dissolving in distilled water. Ten pairs of 5-d-old adults were placed into a round plastic container that contained a vial of tannin solution, a few soybean seeds, and peanuts. In the control treatment, a distilled water vial was provided instead of tannic acid solution. Survival of females and males and the numbers of eggs oviposited were determined each day up to 33 d after infestation when all adults in 1 and 3% tannin treatments had died. The abdomens of dead females were dissected on the day of death to assess the numbers of developed eggs in the ovaries. Tannin solutions were prepared once a week and kept refrigerated at 4°C until use. Salminen and Lempa (2002) showed that the oxidation of hydrolyzable tannins painted on birch leaves was negligible 24 and 48 hr after application. Since our solutions were kept cold in tightly capped

vials, we expect they were stable. Tannin solution vials were changed every other day. This experiment was done in triplicate.

RESULTS

Contents of Tannin in Persimmon Fruits. Tannin contents were measured in astringent (cv. Chongdosi) and sweet (cv. Fuyu) persimmons from June 10 to September 30 in 2001 (Figure 1). In sweet persimmon fruits, soluble tannins

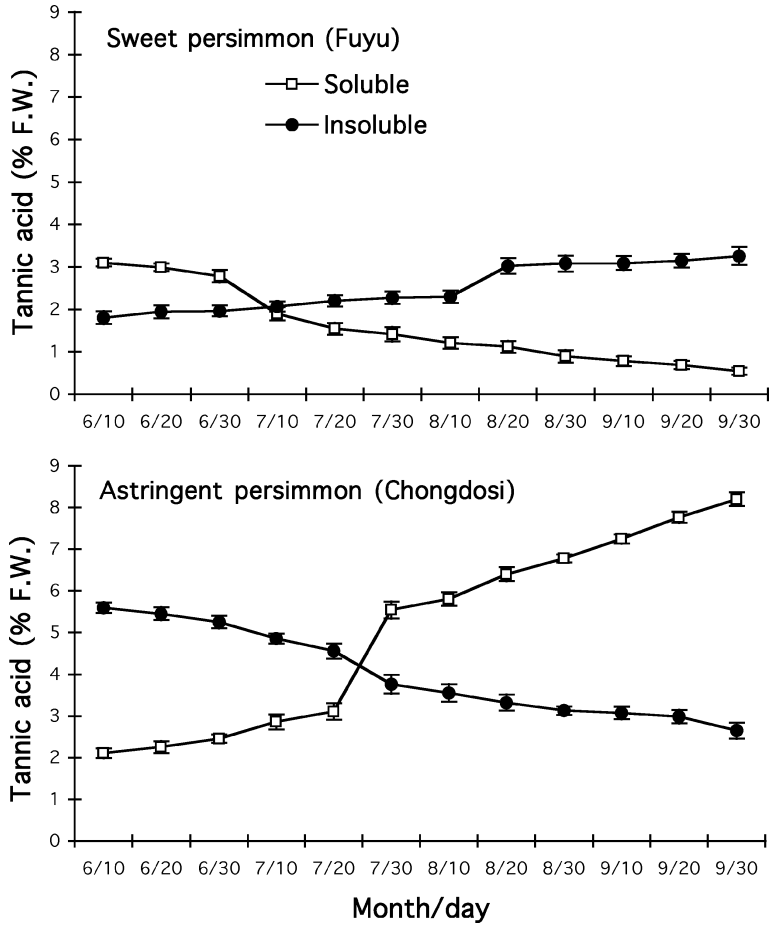

FIG. 1. Seasonal changes in persimmon tannin content (% fresh weight ± SD) of sweet and astringent persimmon fruits expressed as equivalent tannic acid.

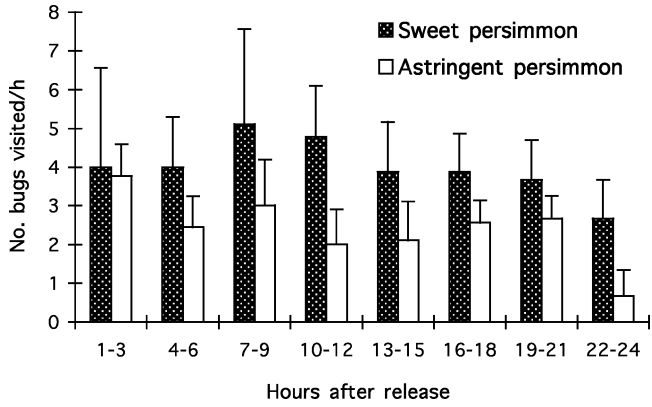

FIG. 2. Number (+SE) of female *Riptortus clavatus* adults that visited persimmon fruits within a plastic cage over a 24-hr period.

decreased gradually from 3.1% on June 10, to 1.2% on August 10, and 0.5% on September 30. The opposite occurred in the astringent persimmon fruits, where soluble tannins increased throughout the growing season, from 2.1% on June 10, 5.8% on August 10, and 8.2% on September 30.

Feeding Behavior of Adult Female Bugs on Astringent and Sweet Persimmon Fruits. The presence of adult female bean bugs was recorded on astringent and sweet persimmon fruits. Over a 24-hr period, the number of females was generally higher on sweet than on astringent persimmon, but not significantly so (t-test, df $= 4$; $P = 0.207$) (Figure 2). Total numbers of piercing/sucking spots were 35 on sweet persimmons and 5 on astringent ones. They are significantly different (t-test, df $= 4$; $P = 0.005$) between sweet and astringent persimmons.

Effect of Persimmon Tannin on Survival of Bean Bugs

Relative Amount of Water and Persimmon Tannin Ingested. Since it was impossible to directly measure the absolute amount of persimmon tannin ingested by the bean bug adults, we used a relative value based on the amount of material excreted onto filter paper (see Methods and Materials). This reflected the amount of water ingested by the bugs. By multiplying this with the corresponding tannin concentration, the relative amount of tannin ingested was obtained. Weights of filter paper were significantly different for bugs fed different persimmon tannin concentrations (Duncan's multiple range test, df $= 10, 4$; $F = 17.1$; $P = 0.001$) (Figure 3). When the bugs were provided with 0.1% tannin solution, they ingested the same amount of the solution as the distilled water control, and it reached 47 times the amount of the no water treatment. At higher concentrations, the amount

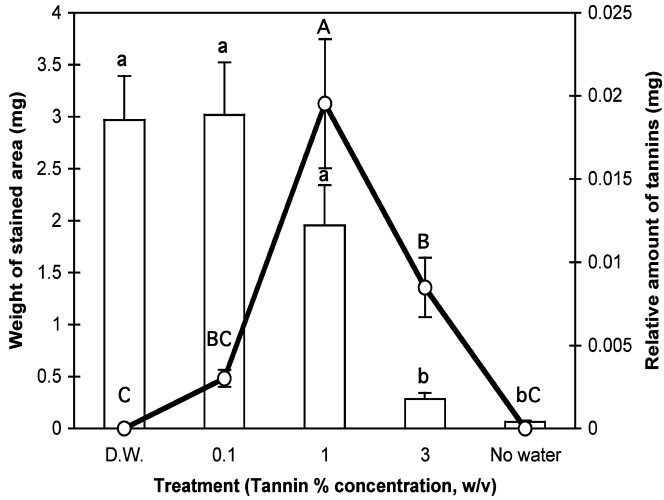

FIG. 3. Weight of filter paper (mg ± SE) stained by excretion (histograms) and relative amount (mg ± SE) of tannins ingested (line) by a bean bug, *Riptortus clavatus*, adult fed different concentrations of persimmon tannin per day. The relative amount of tannins was calculated by multiplying the weight of the area of filter paper stained by excretion with the tannin concentration of each corresponding treatment. The different letters on the histograms or on the line indicate significant differences between treatments (Duncan's multiple range test at $\alpha = 0.05$).

ingested sharply decreased with increasing concentrations of tannin solution. At 1 and 3% treatments, the weights of filter paper were 1.9 and 0.28 mg, respectively, representing 65.8 and 9.5% of those in the distilled water treatment. In the no water treatment, the bugs excreted only on the first day of the experiment. This means that water consumption at high (3%) concentration of tannin is very low, possibly because of poor palatability. The relative amount of persimmon tannin ingested by the bugs was significantly higher at the 1% tannin solution than at the other concentrations (Duncan's multiple range test, df = 10, 4; $F = 18.24$; $P = 0.001$). Even though they ingested a large amount of the solution at the 0.1% treatment, the relative amount of tannin ingested was much lower (15.5%) than at the 1% treatment. When fed the 3% tannin solution, the bugs ingested 2.7 times more tannin than with the 0.1% treatment. Bugs fed 1% tannin solution ingested 6.5 times more tannin than with the 0.1% treatment.

Effect of Persimmon Tannin on Survival and Fecundity of Bean Bugs. Different concentrations of tannin solution were supplied for 33 days to the adults of bean bugs. Persimmon tannin at concentrations of 0.1% had no effect on the survival of female and male adults until day 23 (Figure 4). However, beyond this time survivorship decreased sharply compared with the distilled water treatment.

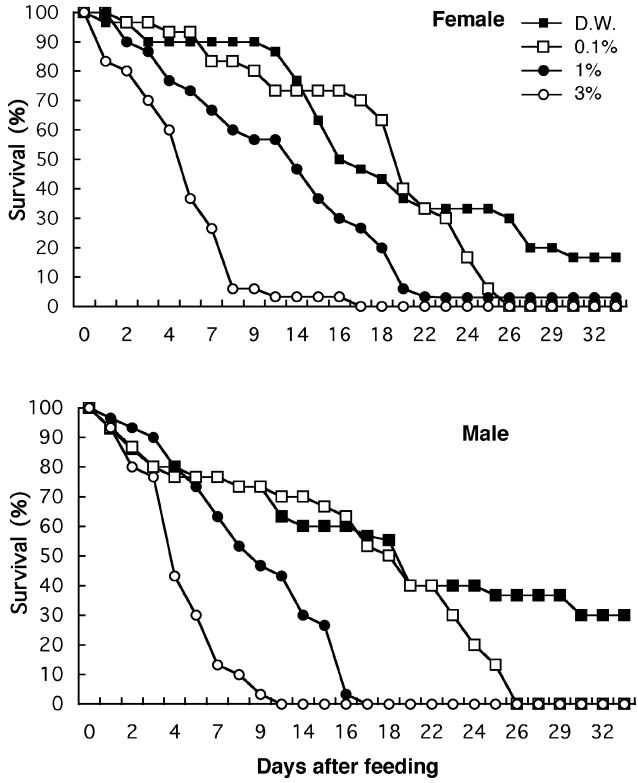

FIG. 4. Effect of purified persimmon tannin on the survival of bean bug, *Riptortus clavatus*, adults at different concentrations (% w/v).

It decreased more markedly in 1 and 3% treatments than in 0.1% or distilled water treatments. When 1% tannin solution was supplied to the bugs, 50% of them were dead at day 14 for females and at day 8 for males. The effect on survivorship was most striking with 3% tannin solution. When responses of both sexes were pooled, 50% mortality was achieved at days 11 and 4 after treatment at 1 and 3% solutions, respectively.

The numbers of eggs oviposited by female adults fed persimmon tannin solutions were lower at the higher concentrations of 1 and 3%. However, 0.1% of persimmon tannin had no significant effect on the egg numbers (Table 1).

Effect of Tannic Acid on Survival and Fecundity of Bean Bugs

Effect on Nymphs. Tannic acid solutions had strong effects on the nymphs (Figure 5). Their survival was negatively proportional to the concentration of

TABLE 1. EFFECT OF PURIFIED PERSIMMON TANNIN ON REPRODUCTION OF BEAN BUG, *Riptortus clavatus*

Tannic acid (%)	No. of females	Total no. of eggs/female/day for 20 days[a]
0.0	30	14.7 ± 5.7 a
0.1	30	12.2 ± 7.6 a
1.0	30	4.6 ± 4.0 ab
3.0	30	0 b

[a] Means followed by the same letter do not differ significantly (Duncan's multiple range test, $\alpha = 0.05$).

the solution. Nymphs began to die on day 2 in 1 and 3% treatments, and on day 4 in the 0.1% treatment. Analysis of variance showed that the survivorship among treatments was significantly different from the 3rd day of the experiment (Duncan's multiple range test, df = 8, 3; $F = 5.79$; $P = 0.021$). Half of the tested nymphs died within 8, 5, and 4 days in the 0.1, 1, and 3% tannin concentrations, respectively. All nymphs were dead at 14, 12, and 7 days at the 0.1, 1, and 3% tannin.

Effect on Adults. Survivorship of bean bug adults was also affected by tannic acid concentration (Figure 6). Responses were similar in both sexes, with higher mortalities occurring at higher concentrations. When the responses of both sexes were pooled, there was no difference in survivorship among control (distilled water), 0.01, and 0.1% tannic acid concentrations. However, 1 and 3% solutions had strong effects on the adults. Survivorship decreased to 50% at days 11 and 6 for the 1 and 3% tannic acid solutions, respectively, and all adults were dead at

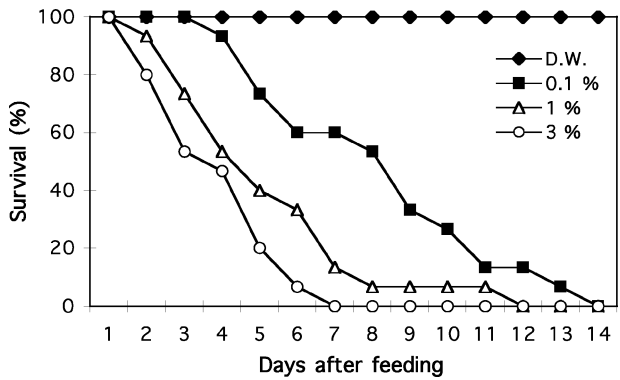

FIG. 5. Survivorship of *Riptortus clavatus* nymphs fed with distilled water or with different concentrations of tannic acid (% w/v).

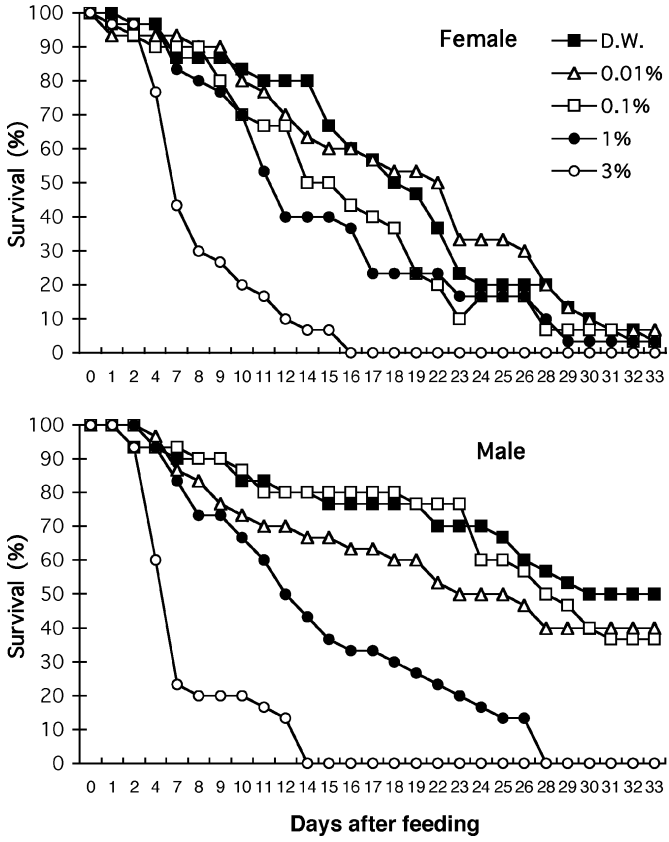

FIG. 6. Changes in survival of bean bug, *Riptortus clavatus*, adults fed with different concentrations of tannic acid solution (% w/v).

days 16 and 29. These results show that the adults are more tolerant to tannic acid solutions.

Effect on Fecundity. The total numbers of eggs oviposited daily by the surviving females for the period of 33 days are shown in Table 2. Numbers of eggs in 0, 0.01, and 0.1% treatments were not significantly different. However there was a significant drop to 4.8 and 0.2 eggs in the 1 and 3% treatments, respectively. Total number of eggs deposited by all females during experimental period was lower at higher concentrations of 1 and 3% tannic acid solution. These might be the result of a sharp increase of mortality in those treatments as shown in Figure 6. Numbers of eggs in ovaries of the dead females were also significantly lower at the higher concentrations of 1 and 3%.

TABLE 2. EFFECT OF TANNIC ACID ON REPRODUCTION OF BEAN BUG, *Riptortus clavatus*

Tannic acid (%)	*N*	Total no. of eggs/female/day for 33 days[a]	No. of developed eggs in ovaries/female	
			No. of females dissected	No. of eggs[a]
0	30	44.8 ± 20.8 a	29	4.8 ± 1.4 a
0.01	30	34.6 ± 11.7 a	28	5.4 ± 1.0 a
0.1	30	32.8 ± 22.4 a	29	3.8 ± 0.7 a
1.0	30	4.8 ± 3.6 b	29	1.9 ± 1.1 b
3.0	30	0.2 ± 0.3 b	30	0.1 ± 0.1 c

[a] Means followed by the same letter in the same column do not differ significantly (Duncan's multiple range test, $\alpha = 0.05$).

DISCUSSION

Major species of bugs on sweet persimmon in Korea are *Plautia stali*, *Halyomorpha halys*, and *Riptortus clavatus*, which suck fruit juice (Chung et al., 1995; Lee et al., 2002). Even though sweet persimmon trees begin to bear visible fruits from late May, *R. clavatus*, according to light trap data, invade sweet persimmon orchards from late June and reach their peak occurrence in late August (Chung et al., 1995). We assumed that content of soluble tannin in the fruits was responsible for the late invasion of the bugs to persimmon orchards.

In preference tests between astringent and sweet persimmon, higher numbers of female adults of *R. clavatus* visited sweet persimmon fruits, and the total number of piercing/sucking spots was significantly higher on sweet compared to astringent persimmon. In this experiment, we used fruits that had been stored at low temperature after harvest. The content of soluble tannin in sweet (cv. Fuyu) fruits at harvesting time is almost 0% (w/w) (Inaba et al., 1971; Ha and Ha, 1993; Taira, 1996). In our analyses, soluble tannin content was less than 0.5% in sweet (cv. Fuyu) persimmon and more than 8% in astringent (cv. Chongdosi) persimmon. This higher content of soluble tannins could be responsible for the nonpreference to astringent persimmon.

High concentrations of tannic acid solution (above 0.1% w/v) affected the survival of nymphs of *R. clavatus*. Nymphs began to die from the 4th day at 0.1% tannic acid solutions. Adult bugs were more tolerant. Concentrations below 0.1% persimmon tannin as well as tannic acid had no effect on adult survival and fecundity. However, they were equally harmful to females and males at 1 and 3%. In the two experiments using tannic acid and persimmon tannin, adult survivorship decreased to 50% at 11 days and 4 or 6 days at 1 and 3% tannin solutions. Decreased survival at higher concentrations could be due in part to the direct effect of tannin ingested by the bugs, and in part to the lower uptake of water in tannin solutions as a result of their unpalatability. Bugs fed with 1 and 3% tannin solution ingested 6.5 and 2.7 times more tannin than those in the 0.1% treatment,

respectively. They also ingested only 65.8 and 9.5% of the water ingested by bugs in the distilled water treatment. Although we are not aware of any published work on the effects of plant tannins on hemipterans, there are many examples showing that plant tannins have harmful effects on development or survival of other insect species. Growth of the winter moth, *Operophtera brumata*, is correlated with tannin levels in oak leaves (Feeny, 1968); increase of the content of oak leaf tannin during the summer may render the leaves less suitable for insect growth by influencing leaf palatability for larvae (Feeny, 1970). In other larvae, such as *Heliothis virescens* (Navon et al., 1993), *Malacosoma disstria* (Lepidoptera) (Karowe, 1989), the aquatic *Acentria ephemerella* (Pyralidae) (Walenciak et al., 2002), and *Aedes aegypti* (David et al., 2000), plant tannins have negative effects on consumption, development, and eventually survival. As in our study, there are a number of examples that the effects of tannins are proportional to their concentration (Rhoades and Cates, 1976; Scriber and Ayres, 1988; Bernays et al., 1989; Schultz, 1989; Clausen et al., 1992; Feeny, 1992; Nomura and Itioka, 2002).

Content of soluble tannin in our analyses dropped to less than 1% in late August. Inaba et al. (1971) reported that the soluble tannin content in Fuyu fruit decreased dramatically after mid-August, reaching around 0.2% in late August. The difference between the two studies could be due to geographical differences between Korea and Japan. However, the same tendency in soluble tannin decrease is reflected in both studies. Sweet persimmon fruits in late August may not be fully palatable to the bugs in terms of soluble tannin content, even though *R. clavatus* adults reach their peak levels at this time in persimmon orchards. However, the sugar content may affect palatability of the fruits. Contents of glucose and fructose in Fuyu persimmon fruits gradually increase from about 10 mg/g (fresh weight) at the flowering stage to more than 100 mg/g at harvesting time (Inaba et al., 1971). In general, carbohydrates such as glucose, fructose, and sucrose are feeding stimulants for insects (Boo, 1998). Because feeding preferences of a species are affected by a number of substances in the food, various concentrations of mixed solutions containing sugars and tannins should be examined for their effects on feeding preference and survival of the bug species that feed on sweet persimmon fruits.

Fecundity of female bugs was strongly affected by higher concentrations of tannic acid and persimmon tannin (1 and 3%). The reasons for reduced survival and fecundity could not be determined from our study. However, there is a possibility that high amounts of tannic acid and persimmon tannins ingested in the 1% treatment acted adversely to *R. clavatus* through a variety of mechanisms. It is now recognized that tannins can produce effects such as inhibition of feeding, reduction in the efficiency of utilization of nutrients, and formation of lesions in the epithelial layer of the midgut (Barbehenn and Martin, 1994). It would be interesting to examine the physiological mechanism(s) involved in the reduced survival and fecundity of *R. clavatus* fed tannin solutions. The reduced amount

of water taken by adult bugs because of the poor palatability of the 3% tannin solution could be another reason for their reduced survival and fecundity.

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CONDURITOLS AS OVIPOSITION STIMULANTS FOR THE DANAID BUTTERFLY, *Parantica sita*, IDENTIFIED FROM A HOST PLANT, *Marsdenia tomentosa*

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Abstract—Host-plant chemicals responsible for egg-laying by the chestnut tiger butterfly, *Parantica sita*, were identified from one of its hosts, *Marsdenia tomentosa*. Ovipositing females responded positively to a methanolic extract of the plant. Solvent partitioning of the extract and oviposition bioassays indicated that the active principle resided in the aqueous fraction. Further activity-directed fractionation of the water-soluble constituents by various forms of column chromatography led to the isolation of several saturated and unsaturated cyclitols together with their glycosides. Of these, conduritol A, a predominant cyclitol present in the plant, moderately stimulated oviposition, while conduritol F 2-*O*-glucoside, although present in a very small amount, evoked a stronger response from females than conduritol A when tested at the same dose. In contrast, its aglycone, conduritol F, which was also a trace component, was almost inactive by itself. However, the oviposition-stimulatory activity of conduritol A was significantly enhanced when tested in combination with a small quantity of conduritol F. Addition of a small quantity of conduritol F 2-*O*-glucoside to conduritol A resulted in a substantial elevation in female responses. Consequently, the synergistic action of a large amount of conduritol A and small amounts of co-occurring conduritol F and its glucoside can account for the stimulation of egg-laying by *P. sita* on *M. tomentosa*.

Key Words—Oviposition stimulants, conduritol A, conduritol F, conduritol F 2-*O*-glucoside, synergism, *Parantica sita*, Danaidae, *Marsdenia tomentosa*, Asclepiadaceae.

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INTRODUCTION

A majority of lepidopterans are phytophagous, but most of them can accept only a narrow range of plant species. Limitations in diet breadth are thought to be mostly due to phytochemical constraints on preimaginal development (Dowd et al., 1983; Terriere, 1984; Berenbaum, 1991; Feeny, 1991) and to a limited capacity for processing neural inputs in the central nervous system (Bernays, 2001). For specialist lepidopterans, accurate recognition and assessment of potential hosts by ovipositing females are, therefore, of prime importance for the successful survival of progeny.

As with larval feeding, host selection by lepidopterous females is governed largely by chemical attributes of plants (Renwick and Chew, 1994; Honda, 1995; Honda and Nishida, 1999). In butterflies, females assess the suitability of a plant they alight on usually by drumming on the leaf surface with foretarsi, which are endowed with chemotactile sensilla that can perceive plant chemicals. Publications dealing with host and nonhost chemicals regulating the acceptance or rejection by ovipositing females have documented a number of oviposition stimulants and deterrents to papilionid, pierid, danaid, and nymphalid species (Honda and Nishida, 1999).

Butterflies in the family Danaidae exploit diverse plant species in the Asclepiadaceae, Apocynaceae, and Moraceae (Ackery and Vane-Wright, 1984). Among these families, they most extensively utilize asclepiads. To the best of our knowledge, investigations on oviposition stimulants have been reported only for *Ideopsis similis* (Honda et al., 1995, 2001), *Idea leuconoe* (Honda et al., 1997), and *Danaus plexippus* (the monarch) (Haribal and Renwick, 1996, 1998).

Danaid butterflies typically occur in the tropics, with some groups ranging as far as subtropical and temperate regions (Ackery and Vane-Wright, 1984). The chestnut tiger, *Parantica sita*, like the monarch, is unique, because the butterfly migrates northward in spring from the Southwestern Islands of Japan or Formosa to the Main Island (Honshu) of Japan. In late autumn, the third generation of butterflies remigrates southward to the Southwestern Islands, where mating and oviposition take place. However, a considerable number of individuals do not migrate, but stay in Honshu, where they also engage in reproductive activity during autumn. Unlike the monarch, *P. sita* never survives winter as an adult, but hibernates as nondiapausing larvae that continue to feed and grow throughout the cold season. Many asclepiad plants are hosts of the butterfly (Endo and Nihira, 1990). In the Southwestern Islands, two evergreen plants, *Tylophora tanakae* and *Cynanchum liukiuense*, are the major host plants from autumn to spring. In Honshu, the butterfly switches its food seasonally: since the two plants are not distributed in Honshu, larvae primarily feed on a few deciduous plants, *C. caudatum, C. grandifolium*, and *T. aristolochioides*, from spring to autumn in the cool montane region, and nonmigrating populations utilize *Marsdenia tomentosa*

as larval food in winter. *M. tomentosa* is an evergreen vine with heart-shaped foliage usually occurring in broad-leaved forests at relatively low elevations where larvae hibernate on the plant. *M. tomentosa* is the one host plant that is available and, hence, indispensable to overwintering larvae of *P. sita* in Honshu. The plant, thus serves as a domicile to otherwise migrating females and is important for maintaining stable populations of the butterfly in Honshu.

Better knowledge of oviposition mediators in danaids is needed to elucidate the phytochemical and sensory mechanisms underlying their host range evolution. Studies on this question have not yet been conducted on the genus *Parantica*. We examined the chemical constituents of *M. tomentosa* responsible for host recognition by ovipositing females of *P. sita*.

METHODS AND MATERIALS

Insects. Females of *P. sita* were collected in Hiroshima and Kohchi prefectures of Japan, and their offspring were subjected to behavioral bioassays. Larvae were reared on potted *C. caudatum* or *T. tanakae* plants in an air-conditioned room under standard laboratory conditions (16L-8D, 23–24°C) from May to October. In winter, larvae fed with *M. tomentosa* were reared in an outdoor cage under natural conditions, where they were allowed to hibernate. Both sexes of newly emerged individuals were kept together under quasinatural conditions until mating in an outdoor cage (7 × 10 m; height, 3.5 m) equipped with flowers as a nectar source and pyrrolizidine alkaloid-containing plants (*Eupatorium* spp.). Copulation occurred usually 3–4 wk after eclosion. Immediately after copulation, females were transferred to the laboratory and kept in transparent plastic chambers. Females were fed with 15% aq. sucrose solution once daily throughout the experiments.

Bioassay for Oviposition Response. The oviposition behavior of *P. sita* females was somewhat peculiar and typically occurred as follows. A female freely flying in the bioassay chamber would alight on relatively mature leaves of a host plant from time to time. Alighting was followed by rapid drumming on the surface of the foliage with the stub-like forelegs. However, an egg was rarely laid on the first landing. After the first drummings, a female would immediately fly away from the leaf, and usually would repeat this behavior one or two more times. Upon the second, or in most cases the third landing, the butterfly would curl the abdomen to bring the ovipositor in contact with the underside of the leaf, and eventually deposit an egg.

The bioassay method for oviposition was designed in view of these female behavioral characteristics. A heart-shaped green plastic plate (a leaf surrogate, 29 cm^2) treated with a test sample of a given dose was presented to a female. She was permitted free flight in a transparent plastic chamber (30 × 40 cm; height, 26 cm) that was externally illuminated with an incandescent lamp (3500 lux). Experiments were conducted using 3- to 5-wk-old gravid females. Before the

bioassay, females were screened daily to determine if they responded positively to the foliage of *M. tomentosa* and negatively to the ovipositional substrate sprayed with water alone (control). Those that failed to display a normal responsive pattern were discarded. Each test sample dissolved in a proper solvent was applied with a microsyringe to an artificial leaf. The treated leaf, once air-dried, was remoistened by spraying with a small quantity of water. For pure chemicals isolated from *M. tomentosa*, oviposition-stimulatory activities of individual compounds were tested singly or in combination. Evaluation of female responses was basically made in accordance with the criteria given in previous papers (Honda et al., 1997; Nakayama et al., 2002) except that a positive response was defined as the deposition of at least one egg within two to three consecutive alightings on the ovipositional substrate. In each trial (sample presentation), the response of an individual was scored as 100% for actual egg-laying or an equivalent behavior, 50% for half-curling the abdomen after drumming (this behavior took place much less frequently), and 0% for drumming only with no positive response. Trials were replicated more than three times for each individual, and the responses of an individual to a given sample were averaged. For all trials, merely alighting on ovipositional substrates without drumming was not included. Unresponsiveness of the females to the control was confirmed every 3 trials of sample presentation to remove any misleading outcome caused by carry-over effects. Oviposition response to each sample was finally represented as the mean percentage of responses recorded from more than 10 females. Significance of differences in oviposition responses among the treatments was assessed by a *t* test.

Extraction, Fractionation, and Isolation of Plant Materials. Young foliage of *M. tomentosa* (1 kg), collected in Hiroshima Prefecture, was homogenized in methanol (6 l) at room temperature. The homogenate was filtered, and the methanol extract was concentrated *in vacuo* below 50°C. The concentrate, after being dispersed in water, was partitioned with chloroform. The chloroform fraction, once evaporated to dryness, was re-dissolved in MeOH. The aqueous layer was fractionated by column chromatography on porous polymer gel (Diaion HP-20, Mitsubishi Kasei Co. Ltd.), and eluted stepwise with H_2O (fraction 1), 25% aq. MeOH (fraction 2), 50% aq. MeOH (fraction 3), 75% aq. MeOH (fraction 4), and MeOH (fraction 5). Fraction 1 was further chromatographed on ODS gel (YMC gel ODS-A 120-S150, YMC Co. Ltd.), eluted with H_2O, 2% aq. MeOH (fraction 1-6), 10% aq. MeOH (fraction 1-7), and 20% aq. MeOH (fraction 1-8), and the first eluate was re-chromatographed on a charcoal column with increasing concentrations (0–20%) of aq. EtOH to yield five subfractions (fractions 1-1 to 1-5). An aliquot of each fraction, once lyophilized, was stored in 50% aq. MeOH below 0°C until use. Fractions 1-1 to 1-6 were individually subjected to silica gel column chromatography (normal phase) using $CHCl_3$-MeOH-H_2O (20:10:1-20:10:2) or EtOAc-MeOH-H_2O (8:2:1) as an eluent. From the resulting fractions, several cyclitols and their glycosides were isolated and finally purified

by HPLC. Details of identification of these compounds based on spectral information (NMR, FABMS), physical properties, and chemical reactions have been reported previously (Abe et al., 1998). Compounds tested as possible candidates for oviposition stimulants included conduritol A (**1**), (-)-conduritol F (**2**), dihydroconduritol A (**3**), kijolanitol (**4**), (-)-bornesitol (**5**), (-)-viburnitol (**6**), conduritol A β-D-glucopyranoside (**7**), conduritol A 1-*O*-α-D-galactopyranoside (**8**), (-)-conduritol F 2-*O*-β-D-glucopyranoside (**9**), and kijolanitol 1-*O*-β-D-glucopyranoside (**10**). Compounds **1**, **2**, and **9** were present mainly in fractions 1-2 and 1-6, compound **3** in fraction 1-2, compounds **4**, **5**, and **6** in fraction 1-3, compounds **7** and **8** in fractions 1-4 and 1-6, and compound **10** in fraction 1-4. The approximate concentration of compound **1** (conduritol A), which was the most abundant of all constituents isolated, was determined for the living foliage by measuring ^{13}C-NMR signal intensity of two equivalent olefinic carbons (129.1 ppm in D_2O) using pure conduritol A and pyrazine as the internal standard. The rough concentrations of the other conduritols and their glycosides were estimated in a similar manner by comparing their signal intensities with that of a known quantity of pyrazine added. The chemical structures of test compounds are given in Figure 3.

RESULTS

Oviposition Response to Fractions. In preliminary experiments, a methanolic extract of *M. tomentosa* elicited a potent oviposition response from females, indicating that the extract contained a key substance involved in stimulation of oviposition. We tested the solvent-partitioned fractions, and then fractions 1 to 5 derived from the aqueous fraction. Since pilot experiments indicated that definite positive responses were evoked at doses higher than 100 μg/cm^2, all fractions derived from the extract were tested at 100 μg/cm^2.

As is evident from Figure 1, active compound(s) were localized in aqueous media, and those present in fraction 1 seemed sufficient to induce egg-laying. This suggested that highly polar and relatively small molecule(s) were most likely responsible for host recognition.

The next experiment tested the stimulatory activity of eight subfractions (Fr.1-1 to 1-8) prepared from fraction 1. Fraction 1-2 had significant activity, while fractions 1-1 and 1-6 were moderately stimulative (Figure 2). Although some of the other fractions had weak activity, most of the active components were in fractions 1-1, 1-2, and 1-6. However, since each of these fractions was less effective than the original fraction (Fr.1), it is probable that several compounds in the plant concurrently stimulated oviposition. We, therefore, examined the components present in fractions 1-1 to 1-6.

Oviposition-Stimulatory Activity of Individual Compounds. From some of the six fractions, 10 compounds (**1–10**) (Figure 3) were isolated together with

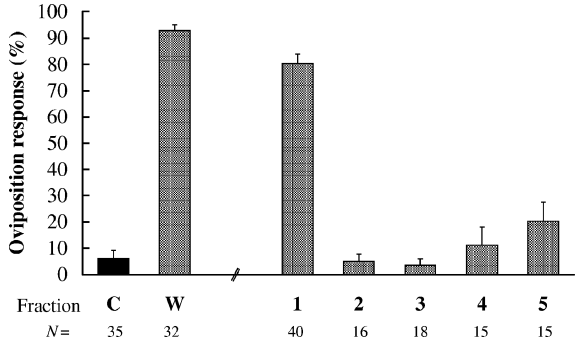


FIG. 1. Oviposition response (mean ± SE) of *P. sita* to fractions derived from a methanolic extract of *M. tomentosa* foliage. C: chloroform-soluble fraction, W: water-soluble fraction. Fractions 1 to 5 were prepared from fraction W. Individual samples were tested at a dose of 100 μg/cm^2.

myoinositol (Abe et al., 1998). Myoinositol was not subjected to bioassay because of its ubiquity in the plant kingdom. The concentration of compound **1** (conduritol A) was estimated at ca. 2% in living leaves of *M. tomentosa*, indicating that the quantity of compound **1** per unit area of the leaf amounts to ca. 100 μg/cm^2. In contrast, rough measurements by NMR revealed that each of the other compounds was present in an amount about 20-fold lower than compound **1**. Thus, the 10 compounds were assayed singly at a dose of 50 μg/cm^2 (Figure 4).

Only two compounds were rated as active; **9** (the strongest) and **1.** All the other compounds, especially saturated penta- and hexa-ols, such as **5** and **6**, were nearly inactive. Although **9** was more than one and a half times as stimulative

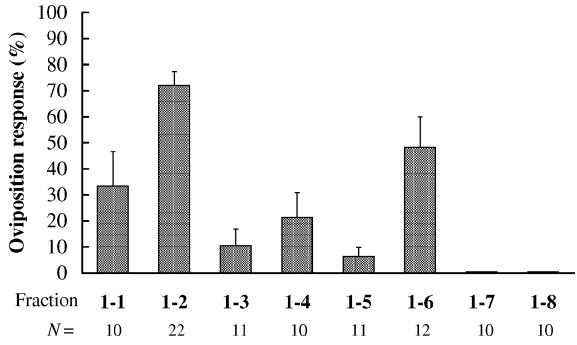


FIG. 2. Oviposition response (mean ± SE) of *P. sita* to fractions prepared from fraction 1. Individual samples were tested at a dose of 100 μg/cm^2.

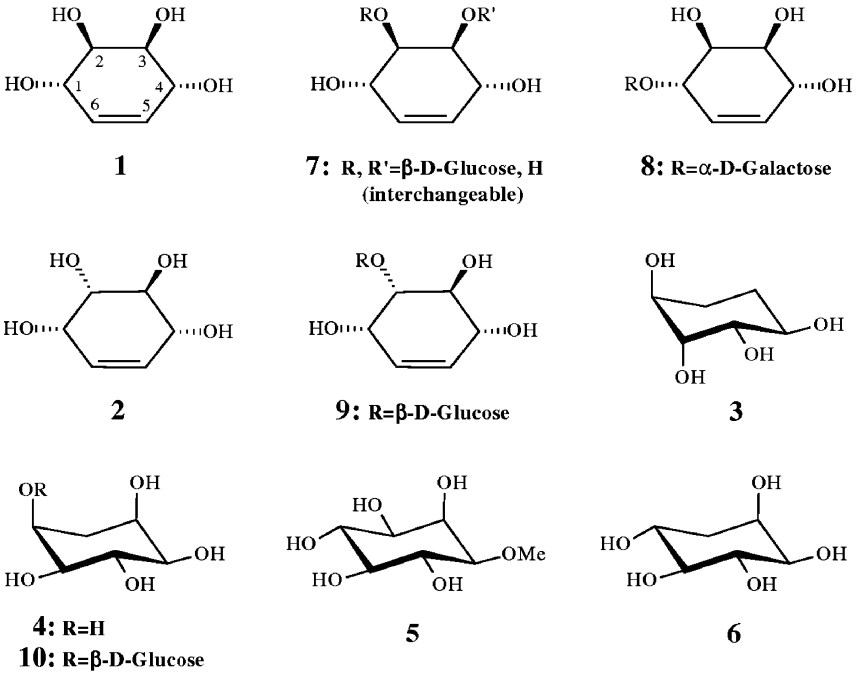


FIG. 3. Chemical structures of compounds from *M. tomentosa* assayed for their oviposition-stimulatory activities. **1**:conduritol A, **2**:conduritol F, **3**:dihydroconduritol A, **4**:kijolanitol, **5**:bornesitol, **6**:viburnitol, **7**:conduritol A glucoside (the position of glucose was considered to be either C-2 or C-3), **8:**conduritol A 1-*O*-galactoside, **9**:conduritol F 2-*O*-glucoside, **10**:kijolanitol 1-*O*-glucoside.

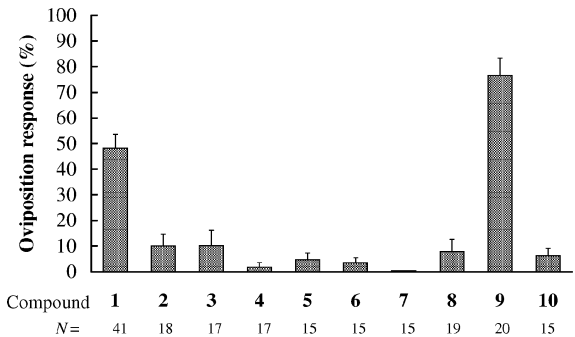


FIG. 4. Oviposition response (mean ± SE) of *P. sita* to cyclitols and their glycosides isolated from *M. tomentosa*. Each compound was tested at a dose of 50 μg/cm^2.

as **1**, the estimated content of **9** in the foliage of *M. tomentosa* was less than 5 μg/cm^2. Therefore, it was unreasonable to assume that **9** at such a low level played a significant role in provoking females to lay eggs on the plant. In fact, a brief oviposition test with **9** performed at 5 μg/cm^2 showed that the compound had no appreciable activity (response: 4.5 $\pm$ 2.5%, $N = 5$). In addition, even when assayed at a much higher dose (100 μg/cm^2), compound **1** only induced a slightly higher response (52.8 $\pm$ 5.6%, $N = 8$) than at 50 μg/cm^2. These findings suggest that some synergistic combination is responsible for eliciting oviposition behavior.

Oviposition Response to Mixed Samples. Some of the minor compounds, selected as potential synergists on the basis of the preceding results, were tested individually in combination with **1** to ascertain whether or not they synergistically stimulated oviposition. In this experiment, females that were much less responsive to **1** were chosen so that the action of each compound could be evaluated more definitely. As shown in Figure 5, **1** evoked almost no positive response at 50 μg/cm^2 (Run 1), although females showed marginally positive responses to the compound at 100 μg/cm^2 (Run 2). Therefore, we examined the activity of test chemicals using admixtures containing a fixed quantity (corresponding to 50 μg/cm^2) of compound **1**.

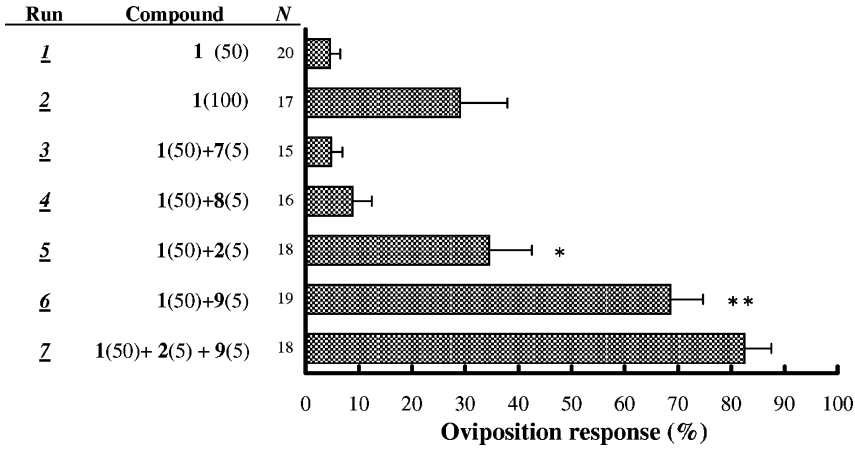


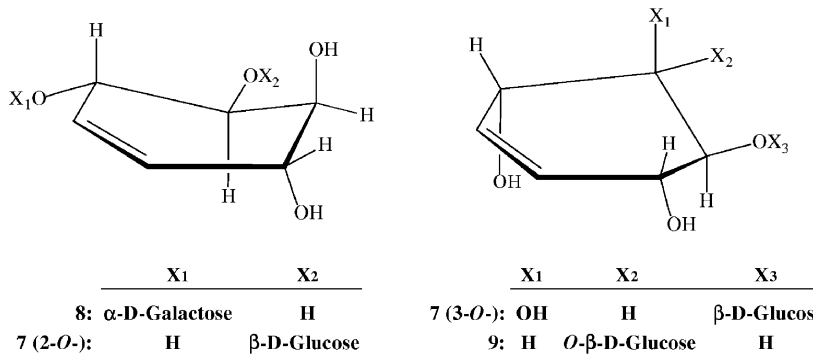
FIG. 5. Tests for synergy of conduritols and their glycosides in oviposition by *P. sita*. The doses (μg/cm^2) at which bioassays were performed are given in parentheses. In this experiment, only females that made little or no positive responses to compound **1** at 50 μg/cm^2 were chosen for the tests (see Run 1). Subsequent follow-up tests showed that when females were used that responded moderately (ca. 40–60% response) to compound **1** at 50 μg/cm^2, the response to the ternary mixture (Run 7) was nearly 100%. The difference in response between Run 1 and subsequent Runs was significant at $P < 0.01$ (*) or $P < 0.001$ (**).

Neither **7** nor **8** augmented the activity of **1** alone (Runs 3 and 4), showing that the two compounds had no synergistic effect on egg-laying. In marked contrast, Run 6 showed that female responses to a mixture of **1** and **9** were far higher than to either alone. A similar, but slightly less prominent result was obtained when a binary blend (**1** plus **2**) was tested (Run 5). Consequently, these data provide unequivocal evidence that both **2** and **9,** while different, stimulate oviposition in synergy with compound **1**. Moreover, a ternary mixture composed of **1**, **2**, and **9**, which approximated the amounts and proportions likely to be experienced by ovipositing females in the field, evoked the highest response (Run 7).

DISCUSSION

Contrary to our expectation, *P. sita* females oviposited in response to highly polar compounds. This is unusual when compared with egg-laying in other danaids. Danaid butterflies in other genera rely exclusively on less polar or nonpolar phytochemicals for host discrimination: Ovipositing females of *Ideopsis similis*, specializing on *Tylophora* plants (Asclepiadaceae), rely on particular phenanthroindolizidine alkaloids (Honda et al., 1995, 2001). *Idea leuconoe*, which is a specialist on *Parsonsia* plants (Apocynaceae), makes use of host-specific macrocyclic pyrrolizidine alkaloids as oviposition stimulants (Honda et al., 1997). Flavonoids (i.e., quercetin glycosides) act as oviposition stimulants for the monarch, *D. plexippus*, which feeds as larvae on a wide variety of *Asclepias* plants (Asclepiadaceae) (Haribal and Renwick, 1996, 1998). The foliage and caules of *M. tomentosa* contain a number of pregnane glycosides, which were isolated from a chloroform-soluble fraction of the plant (e.g., Abe et al., 1999, 2000). The chloroform fraction, however, had almost no effect on *P. sita* oviposition (Figure 1). In fact, a blend of several plant-derived pregnane glycosides did not trigger egg-laying (data not shown). Accordingly, *P. sita* females must recognize *M. tomentosa* as a host simply by examining the composition of water-soluble substances.

Among the cyclitols tested, only three compounds, viz. conduritol A, conduritol F, and its glucoside, were active. Conduritol F, which was inactive by itself, served as a synergist. Since oviposition responses were induced specifically by unsaturated cyclitols (conduritols), it is reasonable to infer that at least one double bond and perhaps a few hydroxy groups adjacent to the double bond are required for stimulatory activity. In addition, comparing the activities between **1** and **2** or **7** and **9**, the stereochemistry at C-2 and *O*-glucosidation at that position, in particular, seem to impact the presence or absence of activity. However, the effect of *O*-glycosidation (or OH-blocking) of aglycones on female responsiveness appears too complicated to explain. Conduritol A completely lost its activity by glycosidation (**7** and **8**). Inversely, conduritol F acquired a striking activity by similar modification (**9**) despite that there is not much difference between **7** and **9** in the



	X1	X2
8:	**α-D-Galactose**	**H**
7 (2-*O*-):	**H**	**β-D-Glucose**

	X1	X2	X3
7 (3-*O*-):	**OH**	**H**	**β-D-Glucose**
9:	**H**	***O*-β-D-Glucose**	**H**

FIG. 6. 3-Dimensional view of conduritols and their glycosides, representing more stable conformers. The position of glucosidation remained undetermined for compound **7**.

rough form of the whole molecule (Figure 6, sugar moieties of stable conformers generally occupy an equatorial position). These results suggest the involvement of more than one highly structure-specific receptor molecule in the perception of active compounds by females.

The natural occurrence of conduritols A and F, and their glycosides has been established (Balci et al., 1990; Desjardins et al., 1999). While conduritol F occurs in almost all green plants at least in traces, the abundance of conduritol A seems restricted to specific subfamilies of the Asclepiadaceae. Certain species in the genera *Marsdenia*, *Dregea*, and *Gymnema* are excellent producers of conduritol A (Kindl and Hoffmann-Ostenhof, 1966; Abe et al., 1998, 2000; Desjardins et al., 1999). Because conduritol A *per se* was not very stimulative even at high doses (Figure 4), it is reasonable to consider that strong responses of females to *M. tomentosa* were evoked not by conduritol A alone, but by the synergistic action of conduritol A, conduritol F, and its glucoside. Of these, conduritol F glucoside plays a significant role (Figure 5), although in the plant tissue it occurred at a level too low for significant activity by itself. In this context, if the plant had contained little or no conduritol A, then females might fail to recognize the plant as a host. In other words, higher levels of the compound might benefit *P. sita* in colonizing *M. tomentosa*.

Assessment of potential hosts based on multiple phytochemical components can be well exemplified by the host recognition system of papilionid butterflies (Nishida, 1995; Nakayama et al., 2003), in which many classes of secondary plant metabolites cooperate in stimulating oviposition. Similar synergism in oviposition occurs in the danaid butterfly, *I. similis*, which relies exclusively on particular phenanthroindolizidine alkaloids for the assessment of host suitability (Honda et al., 2001). However, this mode of synergism contrasts with that of the

papilionids in that oviposition responses of *I. similis* are most effectively induced by the synergistic action of an array of alkaloids with related structures. Obviously, the same situation appears to hold true for *P. sita*, in which a couple of monounsaturated cyclic polyols concurrently stimulate oviposition. In view of the strict discrimination of subtle differences in chemical structures of coexisting cyclitols by ovipositing females and their comparatively versatile ability to recognize molecules of different sizes, host assessment by *P. sita* is likely to be mediated by complex structure-specific receptor proteins of similar nature.

Four danaid butterflies (*I. leuconoe, I. similis, D. plexippus*, and *P. sita*) belong to different genera and utilize distinct (structurally unrelated) categories of compounds as cues when ovipositing. Thus, there appears little or nothing to link the four species together in terms of phytochemicals involved in host selection. Elucidating how they have attained these different sensory systems and host affiliations is a challenging subject worth pursuing.

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THE EFFECTS OF SEED QUALITY AND PIPECOLIC AND DJENKOLIC ACIDS ON BRUCHID BEETLE INFESTATION IN WATER DEFICIT-STRESSED *Acacia* TREES

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Abstract—*Acacia* trees in the Negev desert and the Arava valley of Israel are suffering high levels of mortality due to water stress. Additionally, recruitment is negatively affected by bruchid beetles. We hypothesized that water-stressed trees would be less able to produce secondary defense compounds, such as the nonprotein amino acids, pipecolic acid and djenkolic acid, in their seeds to decrease seed herbivory. We further hypothesized that the high seed infestation reported is due to increased fitness of beetles infesting trees that are in a poor physiological state. Contrary to our prediction, pipecolic acid concentration was higher in water-stressed *Acacia raddiana* trees. We found that infestation rates and beetle fitness were higher in trees in a poor physiological state, despite the higher levels of pipecolic acid in these trees. There was a significant positive correlation between infestation level and the amounts of djenkolic acid in the seeds, indicating that the beetles may have found a means of utilizing djenkolic acid for their own benefit.

Key Words—*Acacia*, bruchid, nonprotein amino acids, water stress, desert, Israel, defense compounds, pipecolic acid, djenkolic acid.

INTRODUCTION

Acacia trees in the Negev and Arava are in substantial danger of extinction due to high mortality levels (up to 60%) and low recruitment (Ashkenazi, 1995; Ward and Rohner, 1997). A major cause of lack of recruitment is infestation of seeds by

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bruchid beetles (Bruchidae) (Ward and Rohner, 1997). Bruchid infestation rates described in the Negev desert for the last 30 years are high. Halevy (1974) recorded rates of *Acacia raddiana*, *Acacia tortilis*, and *Acacia pachyceras* [previously known as *Acacia gerrardii* ssp. *negevensis* (Danin, 1983, 2000)] to be 72, 99, and 64%, respectively. In 1999, Rohner and Ward recorded rates of 97.6% for *A. raddiana* and 96.2% for *A. tortilis*. These infestation rates are much higher than those described for *Acacias* in Africa, which range between 25.2–68% (Lamprey et al., 1974; Coe and Coe, 1987; Miller, 1994; Or and Ward, 2003).

Bruchid beetles (Bruchidae: Coleoptera) are seed pests, mainly on plants of the Leguminosae (Fox and Dingle, 1994). Adult bruchid females lay their eggs on or in *Acacia* pods (depending on the species); the larvae drill into the seed and feed on its contents (Southgate, 1979). The reproductive success of beetles, measured in terms of numbers of infested seeds and numbers of emerging beetles, may be limited by the level of defense compounds produced by the host. *Acacia* tree seeds accumulate nonprotein amino acids as secondary compounds (Seneviratne and Fowden, 1968; Evans et al., 1977, 1993). Of the various nitrogen-based plant toxins, the simplest in structure are the nonprotein amino acids. These are widely distributed in plants and may be directly toxic (see e.g., Romeo, 1998). Romeo (1998), among others, has shown that nonprotein amino acids in mimosoid legumes, such as *Acacia*, may have multiple roles, including adaptation to physical and water stress, insect deterrence and toxicity, and phytotoxicity. Here, we focus solely on the potential antiherbivore properties of these compounds. Nonprotein amino acids may be mistakenly incorporated into protein synthesis causing beetles to produce unnatural and nonfunctioning enzymatic protein. Development is disrupted and death may ensue (Seneviratne and Fowden, 1968; Taiz and Zeiger, 1991). Other nonprotein amino acids interfere with the hardening and darkening of the insect cuticle (Harborne, 1993).

Seneviratne and Fowden (1968) found that the series Gummiferae of the genus *Acacia* is distinctive in its free amino acid content. Among the "uncommon" amino acids in this series was *N*-acetyldjenkolic acid. Pipecolic acid is also found in some species of *Acacia* (Seneviratne and Fowden, 1968; Bradke and Murray, 1989; Kunii et al., 1996). Evans et al. (1977) found the Gummiferae to be characterized by the accumulation of pipecolic and djenkolic acids. Djenkolic acid accumulates in *Acacia* seeds (Shah et al., 1992), and Evans et al. (1993) found *Acacia acapulcensis* seeds contain large quantities of *N*-acetyldjenkolic acid.

Because the *Acacia* populations in the Negev desert and in the Arava Valley are under substantial danger of extinction due to high mortality and low recruitment (Ashkenazi, 1995; Rohner and Ward, 1997), and simulation models show that bruchid infestation has a crucial effect on *Acacia* recruitment (Wiegand et al., 1999), it is important to detect the specific changes, in seed quality that have the most profound effect on bruchid fitness. As defenses against herbivory are costly to the plant (Fineblum and Rausher, 1995; Rohner and Ward, 1997), the ability

to produce herbivore repellents can be predicted to correlate positively with the physiological state of the tree. As the physiological state of the tree worsens, its ability to produce nonprotein amino acids could be expected to decline.

We hypothesized that the high infestation rate of bruchid beetles on *Acacia* trees in the Negev (Halevy, 1974; Ward and Rohner, 1997) is due to the reduced ability of *Acacia* trees in poor physiological state because of water stress to produce nonprotein amino acids. This in turn would allow beetles to grow larger, and to have greater fecundity and survival. This effect on beetle mothers would be passed on to their daughters as an environmentally induced maternal effect (Or and Ward, unpublished data) because of the increased quality of the mothers who invest more resources in their offspring. We analyzed secondary compounds and nutritional factors of *Acacia* seeds as an estimate of seed quality, and inspected the correlation between seed quality, bruchid fitness, and infestation rate. We predicted that bruchid beetles would prefer high-quality seeds. We expressed seed quality in terms of nutritional value (nutrients, proteins, carbohydrates, etc.), and in the scarcity of toxins and repellents (Freeland and Janzen, 1974).

METHODS AND MATERIALS

Bruchid Species (Coleoptera: Bruchidae). The two *Bruchidius* species found on *A. raddiana* and *A. pachyceras* are *B. arabicus* Decelle and *Bruchidius* sp. near *arabicus* Decelle (Anton et al., 1997). Both species complete their development in one seed, emerging as adults. A third species of bruchid beetle, *Caryedon palaestinicus* (subfamily Pachymerinae; Anton et al., 1997) is present in the seeds of *A. raddiana* and *A. pachyceras*.

Estimation of Acacia Physiological State. Acacia physiological state was estimated by measuring tree water potential with a Scholander pressure chamber (PMS Instruments, Corvallis, Oregon) (Scholander et al., 1965). The range of water potentials usually found in vascular plants varies on a diurnal cycle. Immediately before dawn, plants are in a relatively hydrated state (Grace, 1997). Predawn and mid-day water potentials were measured by Shrestha et al. (2003) according to the standard technique of Scholander et al. (1965). We used predawn measurements to assess *Acacia* physiological state because predawn values indicate the maximum water available to a plant. We used trees from the two extremes of *Acacia* water potential range [40 mm (Hai Bar, southern Negev desert, Israel) and 200 mm (Dimona, northern Negev desert, Israel) mean annual rainfall]. Very negative water potential indicated a poor physiological state and *vice versa*.

Nonprotein Amino Acid Analysis. Two grams of intact seeds of *A. raddiana* and *A. pachyceras* were used. Ground seed material was extracted with 70% EtOH for 24 hr with continuous shaking. After centrifugation at 2600 *g* for 5 min, the supernatant was added to a column of Amberlite IR-120 (H^+ form). The column was washed thoroughly with H_2O and the amino acids displaced with 2 M NH_4OH. The

ammoniacal extracts were evaporated to dryness under vacuum at 50°C before resuspension in 1.6 ml of 20 mM K-P_i buffer at pH 7.7. Amino acids were converted to their FMOC-derivatives (9-fluorenyl-methylchloroformate) (Shah et al., 1991). Separation of derivatized amino acids was done on a column of TSK ODS-12OT with a binary gradient solvent elution programme using MeCN and two buffers containing HOAc, TFA, and $(C_2H_5)_3N$. A Perkin Elmer HPLC (high performance liquid chromatography) system was used with a microprocessor-controlled Series 4 quaternary solvent delivery system and an ISS-100 autoinjector, a Nelson PC integrator, and an Hewlett-Packard HP1046A programmable fluorescence detector. Retention times for pipecolic and djenkolic acids were 21.9 min and 15.4 min, respectively. Further details of the methods are outlined in Shah et al. (1991) and Evans et al. (1993).

Nitrogen Content. Two grams of intact seeds were ground in a coffee mill and prepared for analysis by wet digestion with sulfuric acid and hydrogen peroxide, after which nitrogen content was measured using the Nessler method (Allen et al., 1974).

Energetic Value. The energy value of seeds of *A. raddiana* was measured with a standard protocol (see e.g., Allen et al., 1974) using an oxygen bomb calorimeter (Parr Instrument Company, Moline, IL). Benzoic acid was used as a standard. Sources of seeds used in these analyses are listed in Table 1.

Infestation Rate. Infestation rate was measured by taking seeds out of pods and placing them in Petri dishes. One Petri dish per tree was marked for the infestation rate experiment. Marked Petri dishes contained 200 or 250 seeds. All Petri dishes were checked every day for bruchid larvae or adults emerging from

TABLE 1. SOURCES OF SEEDS USED IN ANALYSES OF NONPROTEIN AMINO ACIDS, NITROGEN CONCENTRATION, AND INFESTATION RATES

Species	Site	Mean physiological state of trees	No. of trees
Nonprotein amino acids and energy analyses			
A. raddiana	Paran	Good	7
	Bitaron Wadi	Poor	5
	Bitaron Dyke	Poor	2
A. pachyceras	Tsichor	Variable	6
	Ya'alon	Poor	5
	Milchan	Good	4
Nitrogen content analysis			
A. raddiana	Paran	Good	7
	Bitaron Wadi	Poor	6
Infestation rate measurements			
A. raddiana	Paran	Good	6
	Bitaron Wadi and Dyke	Poor	8

the seeds. *Bruchidius* adults were taken out of the Petri dish, weighed within 24 hr of emergence, put in vials with alcohol, and sorted by tree.

The seeds in each Petri dish were sorted according to the number of bruchid holes in them. They were categorized either as intact, having one to several holes, or as completely damaged (albeit not necessarily by bruchid beetles). We did not differentiate between holes caused by different species of bruchid beetles. Infestation rate was calculated by dividing the total number of holes by the number of seeds, multiplied by 100. Because more than one bruchid beetle can infest a single seed, infestation rate may exceed 100%. Seed sources for these experiments are listed in Table 1.

RESULTS

Nonprotein Amino Acids Analysis. Both *A. raddiana* and *A. pachyceras* seeds contained significantly higher amounts of djenkolic acid than pipecolic acid (ANOVA; *A. raddiana*: $F = 4.927$, $P = 0.036$, error $df = 24$; A. pachyceras: $F = 68.027$, $P < 0.001$, error $df = 28$). The mean amounts (±SE) of pipecolic and djenkolic acids in *A. raddiana* (stressed and unstressed trees) were 536.15 ± 85.36 mg acid/g seed and 738.46 ± 31.94 mg acid/g seed, respectively. The mean amounts (±SE) of pipecolic and djenkolic acids in A. pachyceras were 386.00 ± 25.96 mg acid/g seed and 786.00 ± 40.96 mg acid/g seed, respectively.

There were higher amounts of pipecolic acid in seeds from *A. raddiana* trees in poor *vs*. good physiological state (ANOVA, $F = 5.815$, $P = 0.035$, error $df = 11$) (Figure 1). However, there were no significant differences in the amounts of djenkolic acid between *A. raddiana* trees in poor *vs*. good physiological states (ANOVA, $F = 4.556$, $P = 0.561$, error $df = 11$) (Figure 1).

There was a negative correlation between pipecolic acid concentration and *A. raddiana* water potential ($r = -0.46$, $P = 0.044$, P value obtained by randomization test with 1000 permutations). None of the other correlations (i.e., between pipecolic and djenkolic acids *vs*. water potentials of *A. pachyceras* and *A. raddiana*) was significant.

Nitrogen Content. Trees in good physiological state accumulated more nitrogen in their seeds than trees in a poor physiological state (ANOVA, $F = 10.185$, $P = 0.009$, error $df = 11$). A negative correlation was found between the amount of nitrogen in the seeds and tree water potential ($r = 0.19$, $F_{(1,11)} = 6.026$, $P = 0.032$) (Figure 2).

Energetic Value. There was no significant difference between the energy values of *A. raddiana* seeds from trees in poor *vs*. good physiological states (ANOVA, $F = 0.898$, $P = 0.364$, error $df = 11$). There was no correlation between seed energetic value and tree water potential ($r = 0.28$, $F_{(1,11)} = 0.960$, $P = 0.348$).

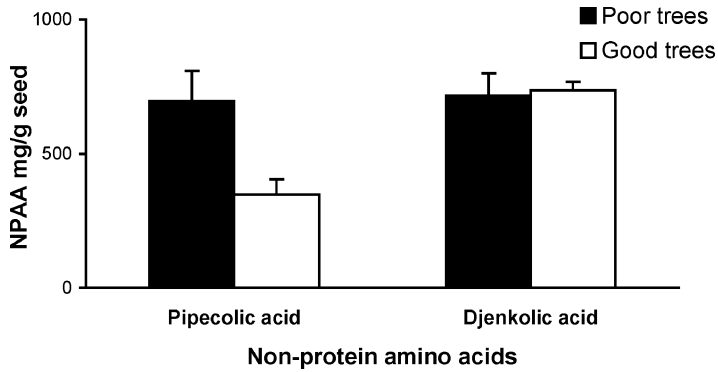

FIG. 1. Mean ± SE amounts of nonprotein amino acids in seeds of *A. raddiana* in poor and in good physiological states. Poor trees had significantly higher amounts of pipecolic acid in their seeds.

Infestation Rates and Correlations with Seed Traits. Trees in good physiological state had more intact (noninfested) seeds (53% intact) than trees in poor physiological state (42.8 % intact) ($\chi^2 = 28.74$, $P < 0.001$, $df = 1$). However, there was no difference in the proportions of seeds with different numbers of holes between trees in good *vs*. poor physiological states ($\chi^2 = 3.782$, $P > 0.75$, $df = 5$).

There was a significant difference between $\log_{10}$ transformed bruchid infestation rates of *A. raddiana* seeds in poor *vs*. good physiological states (ANOVA, $F = 5.321$, $P = 0.042$, error $df = 11$). The mean infestation rates (±SE) of trees

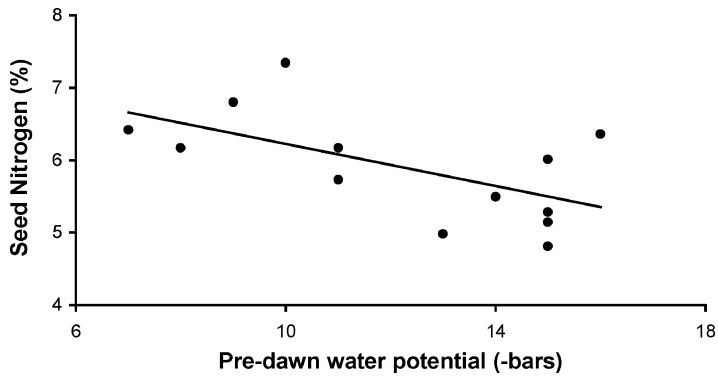

FIG. 2. Correlation between nitrogen concentration (as a percentage of dry mass) in the seeds of *A. raddiana* and tree water stress expressed as predawn water potential. More negative values for water potential is equal to greater water stress.

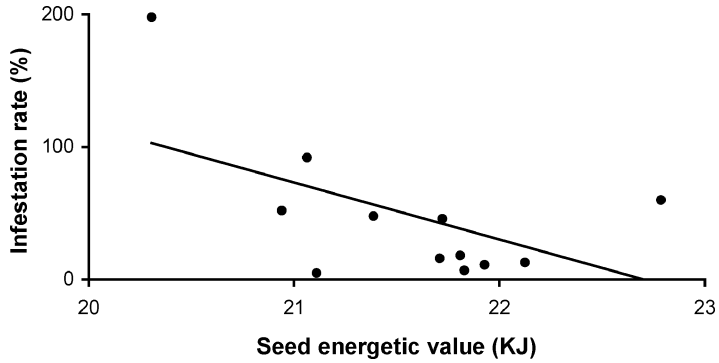

FIG. 3. Correlation between infestation rates and *A. raddiana* seed energetic values.

in good *vs.* poor physiological states were 17.00 ± 6.38 and 67.57 ± 24.15, respectively. However, when one outlier was discarded, there was no significant difference between the two physiological states (ANOVA, $F = 3.859$, $P = 0.078$, error $df = 10$). In this case, infestation rates ($\pm$SE) of trees in good and in poor physiological states were 17.00 ± 6.38 and 45.83 ± 12.45, respectively.

Contrary to our hypothesis, there was a significant positive correlation between bruchid infestation rates and the amounts of djenkolic acid in the seeds ($r = 0.66$, $F_{(1,11)} = 8.320$, $P = 0.015$). There was no correlation between bruchid infestation rates and the amount of pipecolic acid in the seeds ($r = 0.09$, $F_{(1,11)} = 0.087$, $P = 0.774$).

There was no significant correlation between bruchid infestation rates and nitrogen concentration in *A. raddiana* seeds ($r = 0.32$, $F_{(1,11)} = 1.222$, $P = 0.293$). However, there was a negative correlation between infestation rates and seed energetic values ($r = 0.61$, $F_{(1,11)} = 6.342$, $P = 0.029$) (Figure 3).

Bruchid Body Masses and Correlations with Seed Traits. Bruchidius spp. developing in seeds from trees in poor physiological state had higher body masses than *Bruchidius* spp. developing in seeds from trees in good physiological state (Mann–Whitney test, U test statistic $= 36694.00$, $P = 0.017$, $N = 642$). The mean ($\pm$SE) body masses of *Bruchidius* spp. from trees in poor *vs.* good physiological states were 3.18 ± 0.05 mg and 2.97 ± 0.08 mg, respectively. However, there was no significant correlation between the mean *Bruchidius* body mass per tree and *A. raddiana* water potential ($r = 0.31$, $F_{(1,13)} = 1.337$, $P = 0.268$). Furthermore, there were no correlations between mean *Bruchidius* spp. body mass per tree and seed nitrogen content ($r = 0.18$, $F_{(1,11)} = 0.387$, $P = 0.546$), seed energetic value ($r = 0.621$, $F_{(1,11)} = 0.278$, $P = 0.609$), amounts of pipecolic acid in seeds ($r = 0.62$, $F_{(1,11)} = 0.599$, $P = 0.455$), and djenkolic acid in seeds ($r = 0.27$, $P = 0.366$, $F_{(1,11)} = 0.888$). There was also no correlation between infestation rate and *Bruchidius* body mass ($r = 0.62$, $F_{(1,11)} = 0.483$, $P = 0.501$).

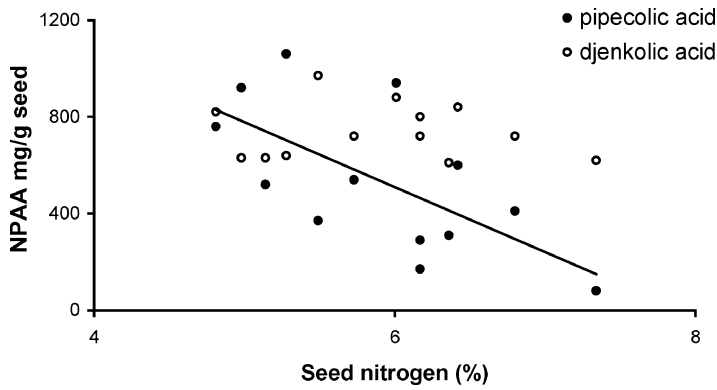

FIG. 4. Correlation between the amount of nonprotein amino acids and nitrogen in *A. raddiana* seeds. The correlation was significant for pipecolic acid only.

Correlations Among Seed Traits. There was a negative correlation between the amounts of pipecolic acid and nitrogen concentrations in *A. raddiana* seeds ($r = 0.65$, $F_{(1,11)} = 8.176$, $P = 0.016$) (Figure 4), but not between djenkolic acid and nitrogen concentration ($r = 0.10$, $F_{(1,11)} = 0.112$, $P = 0.744$) (Figure 4). There was no correlation between the amounts of nonprotein amino acids and seed energetic value (pipecolic acid: $r = 0.13$, $F_{(1,11)} = 0.174$, $P = 0.685$; djenkolic acid: $r = 0.43$, $F_{(1,11)} = 2.514$, $P = 0.141$).

DISCUSSION

Seed Quality Analysis. *A. raddiana* trees in poor physiological state produced more pipecolic acid than trees in good physiological state (Figure 1). It is possible that trees in poor physiological state produce higher levels of secondary compounds in an effort to reduce seed herbivory and, thus, increase their fitness. Because of their poor condition, trees may produce fewer seeds or lower quality seeds due to a trade-off between seed defense and seed number/quality. The production of high levels of pipecolic acid might have been an induced defense that was triggered by herbivory (Karban and Baldwin, 1997). However, responses to herbivory are mostly described for herbivores browsing on leaves, and less frequently on stems and shoots (see Table 4.1 in Karban and Baldwin 1997). An induced response to herbivory on seeds would be less likely to occur because there is no feedback mechanism for the amount of damage caused by the herbivores, because herbivory occurs after dispersal and the entire seed is usually consumed (Rohner and Ward, 1999).

The limited effects of nonprotein amino acids on the bruchids in this study indicate that *Bruchidius* beetles have adapted, at least partially, to detoxify the

secondary compounds and may have found a way of utilizing djenkolic acid for their own benefit, an adaptation resulting in the positive correlation between djenkolic acid and *Bruchidius* infestation rate. Such adaptations to nonprotein amino acids as defense compounds are not unknown. For example, *Caryedes brasiliensis* (Bruchidae: Coleoptera) in Costa Rica feeds exclusively on seeds of *Diocela megacarpa*, which contain more than 8% canavanine (Rosenthal et al., 1976; Rosenthal, 1983), a nonprotein amino acid that is known to retard or prevent cuticle hardening and may even result in death in other species.

Infestation Rate and Tree Physiological State. Trees in good physiological state had a higher proportion of intact seeds. The higher infestation rate of poor trees, and the higher body mass of beetles in poor seeds, may imply that *Bruchidius* spp. developing in seeds from trees in poor physiological state have a higher fitness. Also, larger proportions of intact seeds may indicate that survival in good seeds was lower (holes in *Acacia* seeds are exit holes of dispersing beetles). These results indicate that poor seeds serve as a favorable environment for the bruchid beetles. However, the results of the nitrogen and nonprotein amino acid analyses are inconsistent with this finding because seeds from trees in poor physiological state have lower nitrogen concentrations and higher concentrations of pipecolic acid. Furthermore, there was a negative correlation between infestation rate and seed energy content (Figure 3). In light of the above, we conclude that pipecolic and djenkolic acids produced by *A. raddiana* and *A. pachyceras* do not have a repellent effect on these seed herbivores, in spite of the fact that several studies have shown that these are the most important nonprotein amino acids in African *Acacia* species (Seneviratne and Fowden, 1968; Evans et al., 1977, 1993; Bradke and Murray, 1989; Shah et al., 1992; Kunii et al., 1996).

Pipecolic Acid, Nitrogen, and Acacia Physiological State. There was a negative correlation between the percentage of nitrogen accumulated in the seeds and tree physiological state (Figure 2). Furthermore, there was a negative correlation between the concentration of pipecolic acid and the percentage of nitrogen in the seeds (Figure 4). The latter correlation is compatible with the notion that the synthesis of nitrogenous compounds can create competition for precursors with protein synthesis (Harborne, 1993). The lower the total amount of nitrogen accumulated in the seeds, the higher the proportion of this amount that is invested in pipecolic acid. This result is compatible with the result in Figure 2, showing that trees in poor physiological state accumulate higher amounts of pipecolic acid. The negative correlation between the total amount of nitrogen and pipecolic acid indicates that the production of pipecolic acid is relatively cheap to the tree. As the tree stores larger amounts of nitrogen as condition declines, this is accumulated as pipecolic acid. Interestingly, Romeo and Prass (1980) showed that high concentrations of pipecolic acid in *Ptelea* species (Rutaceae) were found only in species occupying xeric environments. Similarly, Bleecker (1984; reported in Romeo, 1998) showed that *Calliandra* seedlings accumulated more pipecolic acid

in response to drought-imposed physical stress. In plants that recovered upon watering, pipecolic acid levels returned to original levels within days (Bleecker, 1984; see also Romeo, 1998). Romeo (1998) considers the accumulation of cyclic imino acids, such as pipecolic acid, to be a general plant response to a variety of abiotic and biotic stresses (including herbivory). In the absence of strong evidence for an antiherbivore effect of pipecolic acid in the present study, it appears likely that the higher pipecolic acid levels we recorded in seeds from *Acacia* populations in poor physiological condition were induced by water stress.

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COMBINED EFFECTS OF ELEVATED CO_2 AND HERBIVORE DAMAGE ON ALFALFA AND COTTON

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Abstract—We examined herbivore-induced responses of alfalfa (*Medicago sativa*) and cotton (*Gossypium hirsutum*) under different CO_2 conditions. Plants were grown under ambient (350 ppm) or elevated (700 ppm) CO_2 levels, and were either damaged or undamaged by *Spodoptera littoralis* larvae. At harvest, growth of undamaged (control) plants was determined, and foliar chemical composition of both undamaged and damaged plants was analyzed. Cotton grew faster overall and showed a greater increase in growth in response to CO_2 enrichment than did alfalfa. Elevated CO_2 levels increased starch and decreased nitrogen levels in damaged alfalfa and undamaged cotton plants. Alfalfa saponin levels were significantly increased by elevated CO_2 and damage. Regarding specific saponins, medicagenic acid bidesmoside (3GlcA,28AraRhaXyl medicagenate) concentrations were reduced by high CO_2, whereas zanhic acid tridesmoside (3GlcGlcGlc,23Ara,28AraRhaXylApi Za) levels were unaffected by the treatments. Soyasaponin I (3GlcAGalRha soyasapogenol B) was only detected in minute amounts. Alfalfa flavonoid analyses showed that total flavonoid levels were similar between treatments, although free apigenin increased and apigenin glucoside (7-*O*-[2-*O*-feruloyl-β-D-glucuronopyranozyl (1→2)-*O*-β-D-glucuronopyranozyl]-4′-*O*-β-D-glucuronopyranozide apigenin) decreased in CO_2-enriched plants. In cotton, herbivore damage increased levels of total terpenoid aldehydes, gossypol, hemigossypolone, the heliocides H1 and H4, but not H2 and H3, whereas CO_2 enrichment had no effect. These results demonstrate that combined effects of CO_2 and herbivore damage vary between

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plant species, which has implications for the competitive balance within plant communities.

Key Words—CO_2 enrichment, induced response, secondary compounds, cotton, alfalfa, saponins, flavonoids, terpenoid aldehydes, phytochemistry.

INTRODUCTION

Levels of atmospheric CO_2 are on the rise, and this is expected to have a pronounced impact on plant species, communities, and ecosystems. In general, CO_2-enriched plants exhibit increased growth and photosynthetic rate (Bazzaz et al., 1990; Bazzaz and Miao, 1993; Ceulemans and Mousseau, 1994; Curtis, 1996), as well as increased levels of carbon-based compounds, such as primary metabolites and secondary substances (Lincoln et al., 1993; Poorter et al., 1997; Koricheva et al., 1998; Peñuelas and Estiarte, 1998; Agrell et al., 2000). However, plant responses to increased CO_2 levels show enormous inter- and intraspecific variation (e.g., Bazzaz et al., 1990; Lindroth et al., 1993; Kinney et al., 1997; Peñuelas and Estiarte, 1998), which will likely result in an altered competitive balance between species and changes in plant community composition (Peñuelas and Estiarte, 1998; Saxe et al., 1998; Körner, 2000).

Although CO_2 effects vary among plant species, they are also known to be modified by other environmental variables, such as levels of water, nutrients, and light (Bazzaz and Miao, 1993; Kinney et al., 1997; Lawler et al., 1997; Pritchard et al., 1997; Roth et al., 1997; Agrell et al., 2000). A biotic factor that is seriously underrepresented in CO_2 research is herbivore damage. This is surprising considering that CO_2 effects might be largest in the presence of stresses, such as herbivory (Kruger et al., 1998). Herbivore induced responses have been reported for over 100 plant species (Karban and Baldwin, 1997). Since herbivore-damaged plants, similar to CO_2-enriched ones, commonly exhibit altered levels of important secondary substances (reviewed by Karban and Myers, 1989; Karban and Baldwin, 1997), interactive effects between CO_2 and herbivore damage are likely. For example, increased availability of CO_2 may, through enhancement of photosynthesis (Ceulemans and Mousseau, 1994), help defoliated plants to restore an altered source/sink balance after a herbivore attack (Trumble et al., 1993; Kruger et al., 1998). Furthermore, considering what is known about variation in plant responses to CO_2 enrichment, it would be surprising if high CO_2 levels did not also affect herbivore-induced responses of plants. Yet, studies so far have focused almost exclusively on CO_2 effects on undamaged plants, thereby examining only constitutive levels of secondary substances, and only a few have included herbivore damage as an experimental variable. These have focused on interactions between deciduous trees and defoliating larvae, and all have reported responses to herbivore attack to be limited and little affected by

CO_2 availability (Lindroth and Kinney, 1998; Roth et al., 1998; Agrell et al., 1999).

In this study, we compared responses of alfalfa (*Medicago sativa* L.) and cotton (*Gossypium hirsutum* L.) to CO_2 enrichment and herbivore damage. Alfalfa is the world's dominant livestock feeding crop (Small, 1996), and responds to increased CO_2 availability with increased growth, improved tolerance to water stress, and altered phytochemistry (e.g., Sgherri et al., 1998, 2000; Skinner et al., 1999). Cotton is also an important crop species, and like alfalfa, demonstrates increased growth, more efficient water use, and altered foliar chemistry in elevated CO_2 environments (e.g., Reddy et al., 1997; Heagle et al., 1999; Booker et al., 2000). Furthermore, both plant species show herbivore-induced responses. Cotton responds to herbivore damage primarily by substantial increases in levels of terpenoid aldehydes, which effectively deter insect herbivores (e.g., Alborn et al., 1996, McAuslane et al., 1997). The herbivore-induced response of alfalfa was only recently discovered and includes increased foliar levels of saponins and flavonoids (Agrell et al., 2003). To obtain herbivore-damaged plants we used a generalist defoliator, the Egyptian cotton leafworm (*Spodoptera littoralis* Boisduval), which is a major pest on both alfalfa and cotton in the Mediterranean region (Brown and Dewhurst, 1975). Previous studies have demonstrated that feeding by *S. littoralis* larvae induces increased levels of secondary substances in both alfalfa and cotton, and that these in turn reduce larval performance (Alborn et al., 1996; Agrell et al., 2003). Leaves and stems from damaged and undamaged plants, grown in ambient or elevated CO_2 levels, were analyzed for levels of water, starch, and nitrogen, as well as species specific secondary substances. For alfalfa, we analyzed biological activity of saponins, and levels of the specific saponins zanhic acid, medicagenic acid bidesmoside, and soyasaponin I. These dominant saponins in alfalfa aerial parts (Oleszek et al., 1992) are suggested to play an important role in defense against generalist herbivores (Nozzolillo et al., 1997; Oleszek et al., 1999; Adel et al., 2001). Flavonoids also influence insect behavior and physiological performance (Harborne, 1993; Simmonds, 2001), and our analyses included total flavonoids, apigenin (as free aglycone) and 7-*O*-[2-*O*-feruloyl-β-D-glucuronopyranozyl (1$\rightarrow$2)-*O*-β-D-glucuronopyranozyl]-4′-*O*-β-D-glucuronopyranoside apigenin (apigenin glycoside). Cotton secondary substances are dominated by terpenoid aldehydes, which have a large impact on insect herbivores (e.g., Alborn et al., 1996; McAuslane et al., 1997; Anderson and Alborn, 1999). We analyzed total terpenoid aldehyde content, and levels of gossypol, hemigossypolone, and four different heliocides.

The overall aim of this study was to compare alfalfa and cotton with respect to: (1) how growth and phytochemistry are affected by CO_2 enrichment; (2) effects of herbivore damage on levels of nutrients and secondary compounds; (3) plant responses to the combination of elevated CO_2 levels and herbivore damage.

METHODS AND MATERIALS

Plant Growth and Harvesting. Alfalfa (var. Julus) and cotton (var. Delta Pineland 90) seeds were sown individually in plastic pots (1.5 l), and supplied with 2 g slow release fertilizer (Bayer Osmocote, NPK 8-7-16). Plants were grown in climate chambers (3 × 2.5 × 2 m), with environmental conditions similar to those in Southern Europe and the Near East during spring/early summer. The plants, therefore, received a daily horizontal irradiation of 5 kWhr × m^{-2} × d^{-1} (Osram Powerstar, HQI-T, 400 W/D, Daylight), although this was evenly distributed over a 16 hr day (i.e., 620 μmol × m^{-2}× sec^{-1}, with a L16: D8 photoperiod,). Temperatures were 24°C during the day and 20°C at night, with relative humidity 70%. Atmospheric levels of CO_2 were continuously monitored and manipulated in the chambers during the experimental period. The study was performed in four climate chambers, each used once with ambient CO_2 levels (355 ± 6 ppm), and once with elevated CO_2 levels (700 ± 2 ppm, i.e., twice ambient conditions). Thus, the experiment was performed in two consecutive runs. Before the first run, CO_2 levels were randomly assigned to be elevated in two chambers and ambient in the other two. In the second run, CO_2 treatment was switched for all chambers. Six weeks after sowing, the plants within each chamber were randomly assigned to damaged or undamaged (control) treatments. To obtain insect-damaged plants we used *S. littoralis* larvae from a laboratory culture, reared on a semi-synthetic diet (Hinks and Byers, 1976). Two 3rd instar larvae were placed in plastic bags enclosing one stem (alfalfa) or the second true leaf (cotton). The larvae were left to feed for approximately 24 hr, removing 5–10% of the leaf biomass, after which the bags and larvae were removed. Undamaged (control) plants of both alfalfa and cotton had a stem/leaf enclosed by an empty plastic bag, thereby controlling for possible direct effects of the plastic bags. Damaged and control cotton and alfalfa plants were kept together in the chambers until harvested.

Plants were harvested 7 wk after sowing, and plant growth was measured as above ground dry biomass. Each plant was cut at the base of the stem, weighed to obtain fresh mass, put in a paper bag, completely dried (7 d) at 65°C, and re-weighed to obtain dry mass. For each environmental chamber, 12–14 plants of each species were harvested. Only undamaged control plants were included in the growth analysis. Water content (% fresh mass) was calculated from dry/fresh mass ratios for both control and damaged plants. Parallel to whole-plant harvesting, foliar samples were collected for phytochemical analyses. For each chamber and species, samples were collected from five control and five damaged plants. Thus, damaged plants were harvested and analyzed 7 d after herbivore damage. This was because previous studies under similar conditions have shown that both alfalfa and cotton have a defense peak occurring around 1 wk after insect herbivore attack (McAuslane et al., 1997; Anderson et al., 2001; Agrell et al., 2003). Foliar samples

from alfalfa plants were taken from the distal half of all stems except the damaged one, whereas for cotton the two expanding leaves at the top of each plant were collected.

Chemical Analyses. Plant material for chemical analyses was collected 7 wk after sowing. Leaves from alfalfa and cotton were placed in plastic bags, immediately frozen (−40°C), and freeze-dried. The plant material was ground in a Retsch Mixing mill (Wuppertal, Germany), and refrozen (−40°C). Both alfalfa and cotton were analyzed for levels of starch and nitrogen. Starch content was determined by the anthrone method after extraction in ethanol and perchloric acid solutions (Hansen and Möller, 1975), whereas nitrogen analyses were performed with a micro-Kjeldahl-method (see Balsberg-Pålsson 1990).

Biological activity of saponins in alfalfa foliage was determined by the *Trichoderma viride* test. Saponin activity and levels were then indirectly measured as the inhibitory activity of dried alfalfa powder on radial growth of the fungus *T. viride* (Oleszek et al., 1990), which correlates well with haemolytic, phytotoxic, and animal growth-retarding activity (Oleszek, 1993). We also performed detailed analyses of three dominant saponins, zanhic acid tridesmoside, medicagenic acid bidesmoside, and soyasaponin I, using HPLC. Analytical methods followed Nowack and Oleszek (1994). Dried alfalfa powder was extracted in 30% MeOH. The extract was purified using a C18 Sep-Pak cartridge (Waters, Milford, MA, USA), and compounds were determined using a Waters system (Milford, MA, USA) equipped with a Photodiode Array Detector set at 210 nm, and a Eurosphere 80 C18 column (temperature 50°C). The mobile phase consisted of two solvents: A = 1% H_3PO_4 (in water) and B = acetonitrile, and we used a linear gradient from 10 to 90% B. Flow rate was 1 ml/sec. Saponin compounds were identified by comparing their retention times with those of authentic standards and quantified by comparison of their peak area with peak area of the appropriate standard.

Analyses of alfalfa flavonoids included total flavonoid content, free apigenin, and 7-*O*-[2-*O*-feruloyl-β-D-glucuronopyranozyl (1→2)-*O*-β-D-glucuronopyranozyl]-4′-*O*-β-D-glucuronopyranozide apigenin (apigenin glycoside). Following Stochmal et al. (2001), 500 mg alfalfa were refluxed for 1.5 hr in 100 ml 70% MeOH. The extract was concentrated *in vacuo* and purified on a 3 × 5 cm LiChroprep RP-18 column. The column was washed with 40% MeOH, and flavonoids in the MeOH solution were separated on a Eurospher 80 C18 column at 50°C. We used a mobile phase consisting of two solvents: A = 20% AcN in 1% H_3PO_4 and B = 40% AcN in 1% H_3PO_4, and a linear gradient from 0 to 100% B (flow rate of 1 ml/min). Chromatograms were registered and integrated at 350 nm, and calibration curves were prepared using standards of apigenin and apigenin glycoside (Stochmal et al., 2001). To calculate total flavonoid concentration (total integration area at 350 nm) we used the calibration curve of apigenin glycoside.

Methods for extraction and quantification of cotton terpenoid aldehydes followed McAuslane and Alborn (1998). For each sample, 100–105 mg dried plant material were weighed to the nearest 0.1 mg and extracted twice in hexane–ethyl acetate (3:1). The combined organic fraction was dried down to near dryness with N_2, resolved in 2 ml HPLC solvent (19.5% ethanol, 5.4% methanol, 14.2% isopropyl alcohol, 23.6% acetonitrile, 27% water, 4.2% ethyl acetate, 6.0% dimethylformamide, 0.1% phosphoric acid), and filtered through 0.45 μm nylon filters. Samples were analyzed on a liquid chromatograph (Bio-Tek Kontron 525, Milano, Italy) with a 25 cm $\times$ 5 mm C18 column (Spherisorb ODS 2, Chrompack, Solna, Sweden) and a UV-detector (Bio-Tek Kontron 535) set at 272 nm. HPLC was run in the isocratic mode at ambient temperature and a flow rate of 1.25 ml/min. For each sample, double 25 μl injections were made onto the column, and terpenoid aldehydes were quantified in terms of gossypol equivalents using a commercial gossypol standard (Labora Chemicon AB, Sollentuna, Sweden). This also allowed identification of gossypol in the samples via coelution with the gossypol standard.

To confirm identification of the other terpenoid aldehyde components, hemigossypolone, and the heliocides H1–H4, all samples were run against a reference sample (Delta Pineland 90 cotton, grown in the climate chambers) for which the molecular mass of the dominating peaks had previously been established through mass spectrometry. Mass spectrometric analyses were performed on a VG ZabSpec high-resolution mass spectrometer (Fision Instruments, East Grinstead, UK), with an electrospray interface using a hexapole placed before the acceleration path of the ions (cf. Hasselgren et al., 1999). Nitrogen gas was used for drying and nebulizing. Negative chemical ionization was chosen because of its greater sensitivity to terpenoid aldehyde compounds (McAuslane et al., 1997). The electric potential in the ES-interface was: needle voltage 8000 V, counter electrode 5200 V, sampling cone 4200 V, ring electrode 4100 V, hexapole and acceleration voltages 4000 V. The mass spectrometer interfaced with a Phoenix pump (Carlo Erba, Italy), and a 25 cm $\times$ 1 mm C18 column (Kromasil, Bohus, Sweden). The mobile phase was 40% acetonitrile, 40% methanol, and 20% water with a flow rate of 0.1 ml/min. Samples were extracted as described above, and 5 μl were manually injected, with a fixed loop of 20 μl before the column. Commercial gossypol was used as standard and for calibration. Analyzed peaks corresponded to the following molecular masses: 518.60 *m/z* units (to be compared with 518.56 for the gossypol standard), 273.86 *m/z* units (expected for hemigossypolone: 274.08 *m/z* units), and 410.24 *m/z* for two peaks (expected for H1 and H2: 410.20 *m/z* units). Peaks identified as heliocides H3 and H4 after comparisons with previously published chromatograms (Stipanovich et al., 1988; Altman et al., 1990) could not be examined separately in the MS analyses, but lay within a large complex of substances with the molecular mass 410.24 *m/z* units (expected for H3 and H4: 410.20 *m/z* units). One unknown peak in both experimental and reference samples showed no correspondence with previously reported terpenoid aldehydes

(cf. Stipanovich et al., 1988; Altman et al., 1990). Molecular mass of this unidentified component was suggested to be 531.57 *m/z* units, but pending further investigation we will refer to this compound as the unknown terpenoid aldehyde (UTA).

Statistics. Statistical analyses were performed with SPSS 11.5 (SPSS Inc., 2002). To avoid pseudo-replication only the mean value for each experimental chamber was used in the analyses. Thus, for plant growth data, $N = 4$ for each CO_2 treatment, and for chemistry data, $N = 4$ for each CO_2/damage treatment. To correct for heterogeneity of variances, data computed as proportions were transformed (arcsine-square root) prior to statistical analyses. To examine potential differences between chambers, "chamber" was initially included as a random factor in all analyses, but since no significant effects were detected ($P > 0.30$ in all cases), this variable was excluded from the final analyses. Plant growth data were analyzed using a two-way ANOVA, with CO_2 and species as independent variables. Differences in foliar chemistry between treatments were determined for each plant species using a two-way ANOVA, with CO_2 and damage treatment as independent variables.

RESULTS

Elevated CO_2 levels had a strong impact on plant growth (Figure 1). At harvest, 7 wk after sowing, dry mass had increased by over 50% in CO_2-enriched plants (two-way ANOVA, effect of CO_2: $F_{1,12} = 48.8$, $P < 0.001$). At that time, cotton plants were larger than alfalfa plants (effect of species: $F_{1,12} = 167.1$, $P < 0.001$), and also showed a stronger growth response to increased CO_2 availability ($CO_2 \times$ species: $F_{1,12} = 8.29$, $P = 0.014$).

Elevated CO_2 levels decreased water content of both cotton and alfalfa foliage, whereas insect damage had the opposite effect (Table 1). Levels of starch

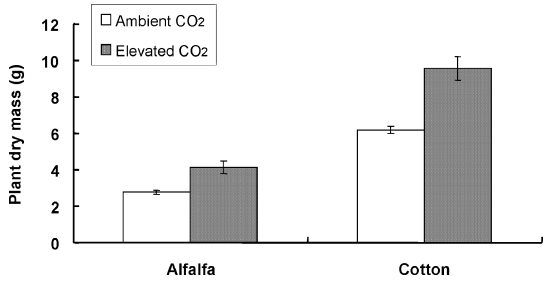

FIG. 1. Dry mass of alfalfa and cotton plants grown in either ambient (355 ppm) or elevated (700 ppm) CO_2 environments for 7 wk. Data are presented as dry above ground biomass. Vertical bars represent SE.

TABLE 1. LEVELS OF PRIMARY METABOLITES (WATER, STARCH AND NITROGEN) IN ALFALFA AND COTTON PLANTS GROWN IN EITHER AMBIENT OR ELEVATED CO_2 LEVELS[a]

Primary metabolite	Ambient CO_2		Elevated CO_2		*P*-value		
	Undamaged	Damaged	Undamaged	Damaged	CO_2	Damage	CO_2 × Damage
Alfalfa							
Water (% of wet mass)	79.8 ± 0.6	81.5 ± 1.0	75.2 ± 0.6	78.9 ± 1.1	0.001	0.005	0.204
Starch (mg/g dry mass)	75.9 ± 13.6	116.5 ± 30.4	54.1 ± 22.4	294.5 ± 84.4	0.088	0.006	0.035
Nitrogen (mg/g dry mass)	42.1 ± 4.3	42.6 ± 3.0	52.1 ± 6.6	28.0 ± 5.6	0.606	0.020	0.016
Cotton							
Water (% of wet mass)	74.4 ± 0.6	80.4 ± 0.5	73.3 ± 0.6	78.3 ± 0.7	0.017	<0.001	0.407
Starch (mg/g dry mass)	195.9 ± 5.1	194.3 ± 17.3	332.8 ± 22.1	204.2 ± 22.5	0.001	0.001	0.002
Nitrogen (mg/g dry mass)	17.5 ± 0.9	21.8 ± 1.2	13.0 ± 0.6	22.3 ± 1.6	0.071	<0.001	0.030

[a] Data (mean ± 1 SE) are presented separately for undamaged plants and insect damaged plants (i.e., plants fed upon by *S. littoralis* larvae for 24 hr 7 d prior to leaf sampling). *P*-values from two-way ANOVA for effects of CO_2 level, damage treatment, and interactive effects of CO_2 and damage are presented.

and nitrogen differed between the two plant species. In alfalfa, foliar starch levels were significantly higher in damaged plants, but this effect was stronger in high CO_2 and more limited in the low CO_2 treatment (i.e., a significant $CO_2 \times$ damage interaction, Table 1). In cotton, starch content was on average increased by CO_2 enrichment and decreased by damage, but like alfalfa, effects of these two experimental variables interacted. Damage had no effect on cotton starch levels in ambient CO_2, but reduced levels by almost 40% in the elevated CO_2 treatment ($CO_2 \times$ damage interaction). Nitrogen content of alfalfa foliage was decreased by damage in elevated CO_2, but not in ambient CO_2 environments. In contrast, cotton nitrogen content was increased by damage overall, although the effect was largest in elevated CO_2. Thus, for both species, the effect of damage on nitrogen was strongest for CO_2-enriched plants ($CO_2 \times$ damage interaction, Table 1).

Analysis of secondary metabolites (i.e., saponins and flavonoids in alfalfa, and terpenoid aldehydes in cotton) revealed some differences between alfalfa and cotton with respect to responses to CO_2 and damage. Only alfalfa secondary compounds were affected by both damage and CO_2 treatments. Biological activity of saponins increased 84% in damaged and 64% in CO_2-enriched alfalfa (Table 2). Of the specific saponin compounds analyzed, levels of zanhic acid were unaffected by the experimental treatments, whereas medicagenic acid bidesmoside content decreased in CO_2-enriched plants. Soyasaponin I (3GlcAGalRha soyasapogenol B) could be detected only in a few samples of damaged alfalfa plants, and was excluded from further analyses.

Levels of total flavonoids in alfalfa were similar between treatments, although effects on specific flavonoid compounds varied. Elevated levels of CO_2 increased free apigenin and decreased apigenin glucoside levels, with somewhat stronger effects in damaged plants (Table 2). Cotton secondary metabolites were instead more consistently affected by herbivore damage. Total terpenoid aldehyde content, as well as levels of hemigossypolone, the unidentified terpenoid aldehyde (UTA), gossypol, and the heliocides H4 and H1 were all significantly increased in damaged cotton plants (Table 2). However, the analysis revealed no influence of CO_2 enrichment on levels of terpenoid aldehydes (Table 2).

DISCUSSION

Alfalfa and cotton differed in response to CO_2 enrichment and herbivore damage. CO_2 enrichment increased growth in both species, but the effect was stronger for cotton than for alfalfa. With respect to phytochemistry, alfalfa responded more strongly to CO_2 enrichment, whereas cotton was more affected by herbivore damage overall. This study, thus, demonstrates significant interspecific variation in plant responses to changes in the environment, which, in turn, has implications for plant–plant competition.

TABLE 2. LEVELS OF SECONDARY METABOLITES IN ALFALFA AND COTTON GROWN IN EITHER AMBIENT OR ELEVATED CO_2 LEVELS[a]

Secondary metabolite	Ambient CO_2		Elevated CO_2		*P*-value		
	Undamaged	Damaged	Undamaged	Damaged	CO_2	Damage	CO_2 × Damage
Alfalfa							
Saponins							
Total saponins (mg/g dry mass)	13.62 ± 2.11	28.42 ± 1.23	25.52 ± 2.16	43.62 ± 4.02	<0.001	<0.001	0.476
ZAT (mg/g dry mass)	6.51 ± 1.53	8.90 ± 2.20	6.49 ± 1.27	7.56 ± 1.52	0.663	0.283	0.676
MAB (mg/g dry mass)	6.22 ± 0.96	5.91 ± 1.35	3.00 ± 0.73	4.67 ± 0.75	0.030	0.461	0.295
Flavonoids							
Total flavonoids (mg/g dry mass)	30.29 ± 2.79	29.55 ± 2.57	25.95 ± 1.45	26.79 ± 2.22	0.101	0.981	0.699
Free apigenin (μg/g dry mass)	84.5 ± 22.6	73.7 ± 19.1	152.0 ± 45.6	280.0 ± 115.1	0.029	0.308	0.232
Apigenin glucoside (mg/g dry mass)	6.08 ± 0.85	8.31 ± 1.07	5.88 ± 0.39	4.84 ± 1.01	0.032	0.447	0.051
Cotton							
Terpenoid aldehydes							
TTA (ng/g dry mass)	2512 ± 627	4183 ± 529	1945 ± 326	4305 ± 185	0.579	<0.001	0.396
HG (ng/g dry mass)	701 ± 117	2140 ± 358	665 ± 98	1922 ± 153	0.496	<0.001	0.622
UTA (ng/g dry mass)	35.8 ± 2.3	90.8 ± 34.8	39.8 ± 9.3	108.6 ± 29.8	0.600	<0.001	0.741
G (ng/g dry mass)	278 ± 102	320 ± 74	228 ± 27	481 ± 47	0.373	0.030	0.102
H4 (ng/g dry mass)	69.1 ± 14.7	225.0 ± 54.3	48.0 ± 5.8	204.9 ± 21.8	0.448	<0.001	0.984
H1 (ng/g dry mass)	113.2 ± 27.6	370.0 ± 73.3	88.8 ± 9.9	396.1 ± 49.7	0.984	<0.001	0.543
H3 (ng/g dry mass)	372 ± 102	324 ± 47	277 ± 44	352 ± 46	0.555	0.813	0.294
H2 (ng/g dry mass)	941 ± 324	711 ± 225	598 ± 221	840 ± 85	0.599	0.976	0.259

[a] Data (mean ± 1 SE) are presented separately for undamaged plants and insect damaged plants (i.e., plants fed upon by *S. littoralis* larvae for 24 hr 7 d prior to leaf sampling). Alfalfa secondary compounds include saponins: total saponins, zhanic acid tridesmoside (ZAT), and 3GlcA,28AraRhaXyl medicagenate (MAB), and flavonoids: total flavonoids, free apigenin and 7-*O*-[2-*O*-feruloyl-β-D-glucuronopyranozyl (1→2)-*O*-β-D-glucuronopyranozyl]-4'-*O*-β-D-glucuronopyranozide apigenin (apigenin glucoside). Cotton secondary metabolites: total terpenoid aldehydes (TTA), hemigossypolon (HG), an unidentified terpenoid aldehyde (UTA), gossypol (G), and the four heliocides H1–H4. *P*-values from two-way ANOVA for effects of CO_2 level, damage treatment, and interactive effects of CO_2 and damage are presented.

Effects of CO_2 Enrichment and Damage on Phytochemistry. CO_2 enrichment and damage influenced foliar chemistry of both alfalfa and cotton, but in different ways in general. Only water levels were similarly affected, with both species in the elevated CO_2 treatment decreasing water content and in the damage treatment increasing water content. A reduction in foliar water levels of CO_2-enriched plants may seem counterintuitive considering that elevated CO_2 levels can improve water-use efficiency of alfalfa and cotton (Reddy et al., 1997; Sgherri et al., 2000). However, this phenomenon has previously been observed (e.g., Lindroth et al, 1993; Lawler et al., 1997; Agrell et al., 2000), and here was probably because increased growth and advanced phenology reduced water content of plants in elevated CO_2 treatments. Interestingly, herbivore damage increased water content. Similar effects of defoliation have been observed for woody plant species (Agrell et al., 1999), and could possibly be because intensive regrowth increase water uptake of damaged plants.

We found increased starch and decreased nitrogen levels in CO_2-enriched plants, and measurable influences of plant species and herbivore damage as well. For alfalfa, elevated CO_2 levels resulted in high starch and low nitrogen content primarily in damaged plants, whereas for cotton, corresponding effects were instead found in undamaged plants. This intriguing interspecific difference may have several causes. CO_2 enrichment often results in high starch and low nitrogen levels (Bezemer and Jones, 1998; Koricheva et al., 1998; Peñuelas and Estiarte, 1998). However, in alfalfa and other legumes, the nitrogen-fixing ability may buffer against CO_2 induced changes of the C/N ratio (e.g., De Luis et al., 1999), which would explain the limited CO_2 effects on undamaged alfalfa in this study. That elevated CO_2 levels were found to increase starch and reduce nitrogen levels in damaged alfalfa plants could be because this buffering capacity was reduced by stress associated with herbivore defoliation. The idea of such interactive effects of defoliation and CO_2 on phytochemistry is in agreement with observations that defoliated alfalfa shows reduced remobilization of nitrogen in elevated CO_2 compared to ambient CO_2 environments (Skinner et al., 1999). Cotton, on the other hand, showed an increase in starch and a decrease in nitrogen in response to CO_2 enrichment only when plants were undamaged. Corresponding treatment effects on damaged plants were lacking probably because cotton shows such a strong response to defoliation (e.g., Karban, 1985; Alborn et al., 1996; McAuslane et al., 1997; Anderson et al., 2001; Agrell et al., 2003). An insect attack commonly triggers a variety of metabolic responses in plants (Karban and Myers, 1989; Karban and Baldwin, 1997; Baldwin, 1999), and substantial phytochemical changes in foliage after herbivore damage most likely eliminated or masked CO_2 effects on damaged cotton plants.

Analyses of the secondary metabolites and biological activity rendered support for the idea that cotton had a stronger response overall to the damage treatment than did alfalfa, which was instead relatively more affected by CO_2 availability.

Insect damage increased biological activity, and probably total levels of saponins, in alfalfa foliage. Increased biological activity is known to correlate negatively with insect performance (Oleszek, 1993). CO_2 enrichment had the same affect, but also increased free apigenin content, and reduced medicagenic acid bidesmoside and apigenin glycoside content. Considering the increased biological activity in damaged alfalfa, we expected increases in levels of medicagenic acid bidesmoside and free apigenin, especially since these compounds have been shown to increase after herbivore attack (Agrell et al., 2003). It is possible that the impact of herbivore damage was relatively limited on these compounds because levels were already relatively high in undamaged plants (cf. Agrell et al., 2003). Another substance that may increase in damaged alfalfa is soyasaponin I (Agrell et al., 2003). This seems to have been the case in the present study, since detectable amounts were found only in samples of damaged alfalfa plants, but levels were overall low. However, the observed increase in biological activity in damaged alfalfa may in part be related to compounds not analyzed in the present study.

In cotton, levels of terpenoid aldehydes were similar in both CO_2 treatments, regardless of whether plants were undamaged or damaged. This study, therefore, shows that CO_2 enrichment had no effect on either constitutive or induced levels of cotton secondary metabolites. Constitutive levels of terpenoid aldehydes have been found to be little affected by CO_2 availability (Lincoln et al., 1993; Koricheva et al., 1998; Peñuelas and Estiarte, 1998), but CO_2 effects on damaged plants have not been previously investigated. The general lack of a CO_2 effect on terpenoid aldehydes is possibly due to the specific biochemical pathways used for synthesis of these compounds (Bezemer and Jones, 1998). For alfalfa, on the other hand, CO_2 enrichment increased both constitutive and induced levels of saponins and free apigenin. Since effects of CO_2 and herbivore damage were additive, the highest levels of these compounds were found in damaged plants in elevated CO_2. These differences between alfalfa and cotton in phytochemical responses to CO_2 enrichment may at least partly explain why cotton showed a stronger growth response to elevated CO_2. Plants are assumed to face a trade off between allocating resources to growth vs. defense (cf. Herms and Mattson, 1992), and it is possible that since cotton does not boost levels of secondary substances in an elevated CO_2 environment, this allows a comparatively large investment in growth.

Ecological Consequences of CO_2 Enrichment and Damage. The secondary compounds of alfalfa and cotton examined in this study are known to have a strong impact on associated herbivores. Alfalfa saponins act as a defense against generalist herbivores (Nozzolillo et al., 1997; Oleszek et al., 1999; Adel et al., 2001). Flavonoids are suggested to have a similar function (Harborne, 1993; Simmonds, 2001), and free apigenin is presumably the active part of flavonoid glycosides. In cotton, terpenoid aldehydes are known to have a strong negative

impact on survival, growth, and development of insect herbivores (Alborn et al., 1996; McAuslane et al., 1997; Anderson and Alborn, 1999). Phytochemical variations observed in this study can be expected to translate into altered herbivore performance, and this also seems to be the case. In a companion study, where *S. littoralis* larvae were fed foliage from damaged and undamaged alfalfa and cotton grown in either ambient or elevated CO_2 environments, the best larval performance (fastest growth and shortest development time) was found on undamaged cotton from ambient CO_2, and the worst on damaged alfalfa from elevated CO_2 (Agrell et al., unpublished data). These data agree with treatment effects on phytochemistry in this study, i.e., low terpenoid aldehyde and relatively high nitrogen levels in undamaged, low CO_2 cotton, and high saponin, high free apigenin, and relatively low nitrogen levels in damaged, high CO_2 alfalfa (Tables 1 and 2). Also, Agrell et al. (unpublished data) showed that for larvae feeding on damaged plants, CO_2 enrichment caused a shift away from alfalfa and towards cotton, again probably a consequence of the "poor" quality of damaged alfalfa in high CO_2.

Taken together, the observed interspecific variation in responses to CO_2 enrichment and herbivore damage has ecological implications. Körner (2000) stated that because plant responses to environmental changes differ, increasing levels of CO_2 is primarily a biodiversity issue. Ecosystem changes in a CO_2-enriched world may occur through interactive effects of an altered competitive balance between plant species and changing population densities of important herbivores (Lindroth et al., 1993; Saxe et al., 1998). The present study confirms that some plant species, e.g., cotton, may gain a competitive advantage because they, relative to other species, show a greater growth response to increased CO_2 availability. However, species showing a less pronounced growth response to CO_2 enrichment may compensate by increasing levels of secondary substances, which would reduce exposure to damaging insects. For alfalfa, this would mean that increased levels of saponins and free apigenin in high CO_2 reduce performance and possibly population size of pest insects, which over time would reduce loss of biomass. However, benefits may also be more direct, since the phytochemical changes in a CO_2-enriched environment have the potential to affect host plant preferences, and direct insect attacks away from alfalfa and towards competing plant species. We conclude that combined effects of CO_2 and insect damage can have an important, but variable, impact on plant species, and have the potential to play an important role in shaping future ecosystems.

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LETTER FROM THE EDITOR-IN-CHIEF

Occasionally, the Journal of Chemical Ecology devotes an entire issue to a single topic. Past instances included the September 2000 issue [Vol. 26 (9)] that focused on Allelopathy and the October 2002 issue [28 (10)] that dealt with Aquatic Chemical Ecology. Plans for a special issue devoted to Molecular Chemical Ecology have been in place for over three years. Early discussions with Linda Walling of the Editorial Board, Associate Editor, Kelsey Downum, and past Associate Editors, Walter Leal and Nancy Targett convinced me that the Journal should aggressively recruit research papers in this area and showcase them in a special issue.

The project stumbled along for a while with little progress. Despite two "calls for papers" and tentative commitments by some to write mini-reviews, we did not make significant headway until earlier this year. It was then that Editorial Board member Steve Seybold agreed to take the lead in soliciting timely reviews, and a number of us actively solicited papers from important laboratories working with molecular tools. By arbitrarily setting the final date for December, 2004, and agreeing to "go" with whatever quality papers we had by September, the issue miraculously came together.

Unlike our goals for special issues in the past—in the case of "allelopathy" to feature the best papers from the International World Congress and to establish guidelines and models for quality papers in an effort to advance the field—and in the case of the "aquatic" issue to showcase important work and to help define the "state of the art" in a sub-discipline just beginning to flourish—the goal this time was different. Our aim here is both to educate our readership about important chemical ecological questions that are being attacked, the answers that are emerging, and the resulting challenges and opportunities that come with molecular work, and also to broaden the scientific base of the papers that the Journal publishes.

A lot of people are acknowledged for their efforts: Steve Seybold for taking the lead; Linda Walling for helping with planning; several past and present Editorial Board members for encouragement including May Berenbaum, Wilhelm Boland, Marcel Dicke, Wittko Francke, Jonathan Gershenzon, Thomas Hartmann, John Hildebrand, Walter Leal, Jocelyn Millar, James Nation, John Pickett, Coby Schal, and Nancy Targett; and finally, many International Society of Chemical Ecology

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members too numerous to mention who served as sounding boards, drum beaters, and reviewers. The Editors are pleased with what has resulted. We hope the readership finds that it was worth our effort.

John Romeo, Editor
Journal of Chemical Ecology

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THE EIGHTH DAY OF DISCOVERY: MOLECULAR BIOLOGY COMES TO CHEMICAL ECOLOGY

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Fifty years ago last spring, the dawning of the molecular age was heralded by the publication of a series of papers on the structure of DNA in the British journal *Nature*. The most famous of which, by J. D. Watson and F. H. C. Crick (Watson and Crick, 1953), has assumed almost mythic proportions. The excitement and promise of the new approach to biology was brought to life for popular culture in Watson's "The Double Helix," and H. F. Judson's "The Eighth Day of Creation." The latter account anchored the roots of molecular biology in the structural chemistry of Sir Lawrence Bragg's Cavendish Laboratory in Cambridge, United Kingdom, and in the phage and bacterial genetics groups led by Joshua Lederberg, Max Delbrück, and Salvador Luria in the United States, and by François Jacob and Jacques Monod in France. During my formative years at the University of Wisconsin, I recall reading these books, learning about the tools of recombinant DNA, and wondering if they might ever be brought to bear on the research problems of agriculture, forestry, and ecology.

The day has now arrived when the tools of molecular biology (Table 1) pervade all levels of biological research and thought, including the field of chemical ecology. Like the old political adage in the United States about "a chicken in every pot," every new biological scientist seems to buy a PCR machine with his or her start-up funds. Research tools such as gel electrophoresis, Southern, northern, and western blotting, PCR, and DNA microarrays are likely to become as familiar and indispensable to the next generation of chemical ecologists as GC-FID, GC-MS, HPLC, and LC-MS have been to the current generation. As a consequence, the interdisciplinary lines between molecular biology and other biological disciplines are beginning to blur. In 1986 at a Steamboat Springs, Colorado meeting,

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TABLE 1. KEY TECHNICAL ADVANCES IN MOLECULAR BIOLOGY

Advance/Technique	Function	Citation
Double-helical DNA structure	Replication of genetic material	Watson and Crick, 1953
Restriction endonucleases	Creation and isolation of discrete DNA fragments	Smith and Wilcox, 1970
Recombinant DNA	*In vivo* cloning of DNA fragments	Cohen et al., 1973; Morrow et al., 1974
Southern blotting	Separation and visualization of specific DNA fragments	Southern, 1975
Northern blotting	Separation and visualization of specific RNA fragments	Alwine et al., 1977, 1979
Western blotting	Separation and visualization of specific proteins	Towbin et al., 1979; Burnette, 1981
Polymerase chain reaction	*In vitro* cloning of DNA fragments	Mullis et al., 1986; Mullis and Faloona, 1987
High throughput sequencing	Rapid decoding of DNA information content	Sanger et al., 1977; Smith et al., 1986
DNA microarrays	Genome-wide analysis of mRNA levels	Schena et al., 1995; Shalon et al., 1996; DeRisi et al., 1997; Eisen and Brown, 1999
Genome sequencing	Cataloging of genome-wide information content	Adams et al., 2000; Lander et al., 2001; Venter et al., 2001

"Molecular Entomology," the *Drosophila* molecular geneticist Michael Ashburner mused that what molecular biologists really needed was a taxonomy and ecology of molecules to help organize all of the signaling molecules, receptors, transcription factors, catalysts, and nucleic acid sequences and their interactions. To some extent, the developments of GenBank and functional genomics, proteomics, and metabolomics have risen to meet Ashburner's call of 20 years ago. Conversely, organismal taxonomy, perhaps our most venerable biological discipline, is itself going through an intense debate about the extent of the role that nucleic acid sequences should play in its future (Hebert et al., 2003; Mallet and Willmott, 2003; Sperling, 2003; Tautz et al., 2003; Will and Rubinoff, 2004).

The *Journal of Chemical Ecology* (*JCE*) published its inaugural issue in 1975, and for several decades has been characterized by research articles dominated by the fertile union of bio-organic chemistry and ecology. The tools of these disciplines have been adequate to tackle the scientific challenges outlined in the *JCE* frontispiece, which are to seek to understand "the origin, function, and significance of natural chemicals that mediate interactions within and between organisms. Such relationships, often adaptively important, comprise the oldest

of communication systems in terrestrial and aquatic environments." *JCE* is the official publication of the International Society of Chemical Ecology (ISCE), a vital, vibrant, and evolving organization that mirrors the scientific interests and approaches of its members.

We are fortunate to live in a revolutionary era of biology in which the basic research questions of chemically mediated interactions among organisms, and between an organism and its abiotic environment, have not changed, but the new molecular tools are transforming the richness and depth of our research accomplishments and increasing the complexity of the questions that we can ask. In her 2000 ISCE Silverstein/Simeone Lecture, Berenbaum (2002) set the tone with her focus on environmental response genes and the role of molecular approaches in comprehending how these genes function in the context of chemical ecology. But silently, and even before Berenbaum's review paper, the *JCE* has been gradually embellished by research articles that are wholly or in-part, molecular. A literature search in September 2004 of the major electronic databases over the lifetime of the *JCE* reveals a clearly increasing trend of publication of "molecular" articles (Figure 1). Beginning in 1986, 55 articles with molecular content, including the 11 articles in this issue, have been published in *JCE*.

Should this development threaten *JCE* and its contributors and patrons? The answer is most certainly no! As Berenbaum (2002) has pointed out, molecular biology is compatible with, if not an essential partner with chemical ecology. The newly coined fields of genomics, proteomics, and metabolomics (see Tittiger, this issue) are, in the final analysis, a systems or a holistic approach to molecular biology with which chemical ecologists are intimately familiar. Metabolomics taken out of the cell is exactly what chemical ecologists have been doing for years. Thus, we find ourselves at the beginning of a great convergence where the molecular and ecological worlds will meet and share their expertise as never before.

In this issue of *JCE*, we offer a collection of scientific articles that capture the impact of the advent of molecular biology on the field of chemical ecology. The content of the articles is admittedly biased towards research on plants and insects, but this may reflect the state-of-the-art in molecular chemical ecology. The four reviews and seven research papers address two general areas where molecular biology is influencing chemical ecology: the physiological or suborganismal level and the organismal (including population and evolutionary biology) level. The definition of chemical ecology (see above) includes both of these levels of inquiry.

Tittiger's review of functional genomics and insect chemical ecology is a primer of high through-put molecular methods written with the lay readers in mind. It is an excellent introductory article for this issue as it emphasizes those versatile modern molecular techniques that can now be applied to virtually any ecologically or economically important, i.e., non-model, organism. Jacquin-Joly and Merlin review how the use of genomics is providing solutions to the formidable problem of

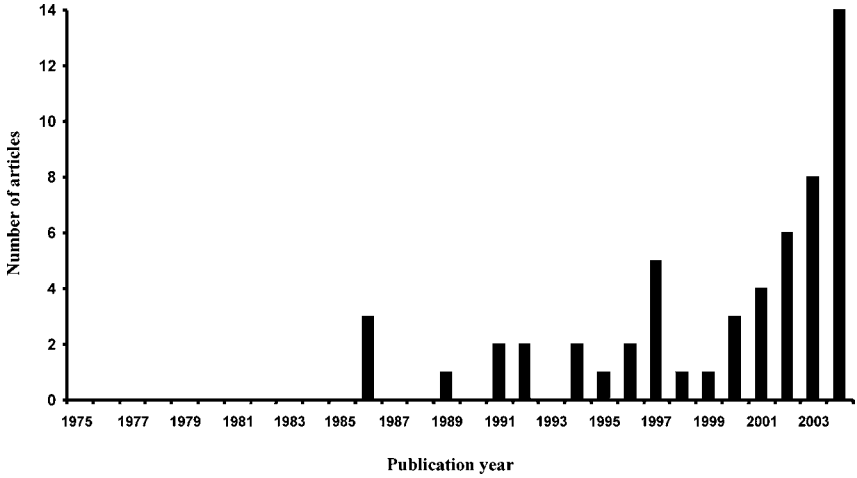

FIG. 1. Publication of articles with molecular content in *JCE* since inception (1975). The following key words and acronyms were used in an "all fields" database search of AGRICOLA, AGRIS, CAB Abstracts, and other databases: AFLP (2, 1), antibody (1, 0), binding assay (0), cDNA (8, 0), cloned (3, 0), cloning (5, 0), DNA (17, 1), enzyme (11, 1), gel electrophoresis (5, 2), gene (20, 2), gene expression (10, 0), genomics (5, 0), isozyme (1, 1), molecular (23, 1), mRNA (3, 0), northern blot (3, 0), PAGE (7, 1), PCR (5, 1), polymerase chain reaction (0), promoter (0), protein (18, 3), proteomics (1, 0), restriction enzyme (0), RNA (3, 0), sequencing (3, 0), Southern blot (1, 0), transcription (4, 0), and western blot (1, 0). The search yielded 55 articles with many redundant hits across all categories. The parentheses after the key words indicate (number of hits, number of hits unique to that field). All article abstracts were reviewed to insure that some molecular technique or research question was covered in the article.

describing the structure and function of membrane-bound proteinaceous olfactory receptors in insects. Already we have learned that one olfactory neuron expresses only one type of olfactory receptor and that there is extreme conservation between insect and vertebrate olfactory systems. This work is enhancing our understanding of the fundamentals of insect chemical communication mechanisms and has ramifications for vertebrate olfaction as well. In plant chemical ecology, Huber et al. describe how the terpenoid defense systems of conifers are dictated by large and functionally diverse terpene synthase gene families. These genes are differentially expressed prior to, during, and following attack by insects or pathogens, and the genomics approach should quickly and comprehensively allow us to catalog these responses in these important plants. Similarly, from a broader perspective, Kaloshian reviews the role of plant resistance genes in the specific recognition of insects or pathogens in the so-called "active" form of plant defense.

The research articles in this issue also bring to the fore the impact of molecular biology on insect and plant chemical ecology. From a physiological or reductionist perspective, Liu et al. describe the isolation and characterization of an insect salivary lysozyme gene from *Helicoverpa zea* using polymerase chain reaction (i.e., *in vitro*)-based cloning followed by Southern, northern, and western blotting to characterize the nucleic acids and the resulting enzyme. Thus, these authors illustrate the application of nearly the full range of molecular tools (Table 1) in isolating this gene and studying its product in relationship to the plant hosts of the lepidopteran pest. Tillman et al. utilize the already-described sequence of a key regulatory enzyme (HMG-CoA reductase) for isoprenoid pheromone biosynthesis in bark beetles to conduct northern blot analyses that align the time- and dose-responses of transcription with those of enzyme activity and metabolic end product (the pheromone components ipsenol and ipsdienol). In the latter study, the analysis reveals differences in the regulation of pheromone biosynthesis related to the phylogeny of the *Ips* spp. Using a subtractive library technique, Lawrence et al. analyze cDNAs comparatively from wounded and volicitin-treated maize plants to isolate plant genes whose expression is increased by the feeding of the beet armyworm, *Spodoptera exigua*. The transcripts, in this instance, are involved in the production of volatiles that attract a parasitoid natural enemy of *S. exigua*. Groot et al. present research that relies on amplified fragment length polymorphism (AFLP) with the DNA of two moths, *Heliothis virescens* and *H. subflexa*, and their hybrids, in conjunction with a classical hybridization genetic analysis to identify the key chromosome that contains elements important in pheromone production. This work combines the direct AFLP assay for the key chromosome with a phenotypic assay of pheromone production in the hybrids, and will ultimately answer fundamental questions about how moths evolve species-specific pheromone communication systems.

The work of Groot et al. is a dual example of how molecular biology can be used to address physiological questions, and to address population-level and evolutionary questions. As another example of the latter line of inquiry, Figueroa et al. use an analysis of the highly variable microsatellite DNA of the aphid, *Sitobion avenae*, to study the genetic variability of its population in relationship to their poaceous hosts. These workers hypothesize that certain rare genotypes persist because of their genetic capacity to detoxify the chemical defenses of the hosts. Florane et al. also use microsatellite DNA to analyze insect populations, but in this case the target is the social organization and differentiation of the colonies of the Formosan subterranean termite, *Coptotermes formosanus*. Cooper et al. examine the interaction of *R*-gene-mediated resistance and the acquired resistance associated with jasmonic acid (JA) and salicylic acid in tomato against the potato aphid, *Macrosiphum euphorbiae*. From a molecular perspective, the expression of a JA-inducible proteinase inhibitor is a key assay that they used to measure resistance against the aphid.

I hope and anticipate that this issue of *JCE* will stimulate the readership to both examine the origins and mission of chemical ecology and to glimpse forward to the exciting scientific outcomes that the grand union with molecular biology will bring to the field. Most of all I hope that the readership will derive as much pleasure and knowledge from the contributions to this issue as I have enjoyed in helping to assemble them.

Acknowledgments—I thank Dr. Dezene P. W. Huber (UC-Davis) for invaluable and untiring assistance with this issue and Kamal J. K. Gandhi and Andrew D. Graves (both belonging to University of Minnesota) for assistance with literature retrieval. I am especially grateful to Majorie A. Hoy (now at University of Florida), and most recently, Claus Tittiger (University of Nevada-Reno) for stimulating my interest in molecular biology, and to David L. Wood (UC-Berkeley) for supporting my enthusiasm for molecular biology early in my career at a time when it was not a fashionable pursuit for an ecologist.

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REVIEW ARTICLE

FUNCTIONAL GENOMICS AND INSECT CHEMICAL ECOLOGY

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Abstract—High-throughput molecular techniques (i.e., genomics) are now beginning to make their way into chemical ecology research. Pioneering functional genomics studies have made significant contributions to our understanding of insect pheromone production, reception, behavior, and insect–plant interactions. Much of this research involves nonmodel organisms, including the honey bee, silkworm, and bark beetles, underscoring that researchers need not be restricted to traditional model organisms for high-throughput research. Furthermore, the technology can reveal physiological interactions that might otherwise be missed by more traditional molecular approaches. Functional genomics should become more widely used as researchers appreciate the wealth of information this potent approach can supply. This review concentrates on a summary of available technologies for functional genomics as they may be applied by chemical ecologists studying insects. Allied technologies (proteomics and metabolomics) are introduced briefly toward the end in the context of future applications.

Key Words—EST, expression profiling, functional genomics, microarray, SAGE, MPSS.

INTRODUCTION

Chemical ecology studies have focused mostly on behavioral and chemical/biochemical methods, with the application of molecular methods appearing relatively recently. Genomics techniques have been rapidly embraced by other disciplines and are beginning to appear in chemical ecology research as well. Functional

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genomics has been used to study prokaryotic chemical ecology (Whiteley et al., 1999; DeLisa et al., 2001) and plant–insect interactions from a plant perspective (Moran et al., 2002; Halitschke et al., 2003; Hui et al., 2003), but very few chemical ecologists have utilized the technologies with insects. This should change as the associated costs decrease, more genomic information becomes available for more species, and as researchers begin to realize the benefits offered by functional genomics.

Terms and Technologies. Standard molecular biology notation will be used throughout this article. Gene names are italicized, while transcripts and encoded proteins appear in normal font. "Transcript" refers to the mature RNA version of a gene, and is used interchangeably with "message" and "mRNA" for genes that encode proteins. A gene's "expression level" refers to the abundance of its corresponding product, and is a function of both transcription and translation. In general, expression levels correlate with transcript abundance, and the two terms are often used interchangeably. However, it is best to distinguish between these terms because numerous mechanisms regulate how actively a gene is transcribed, how efficiently the transcript matures, how long the transcript remains in the cell, and how actively it is translated.

"Genome" means the entire genetic content of a cell/organism, and "genomics" was coined to describe genome research (Weaver, 2002). Functional genomics and structural genomics are different sub-disciplines. Functional genomics studies how the genome is used by the organism, whereas structural genomics describes how the genome is organized. Functional genomics is concerned with what portion of the genome is used under given physiological and/or developmental conditions, as well as how the expressed genes interact with each other. Because the perspective is meant to be comprehensive, the "-ome" suffix is often grafted onto any suitable word by researchers wishing to distinguish their work from historically reductionist approaches. Hence, functional genomics is concerned primarily with the "transcriptome" (the entire RNA content of a system), and sometimes with the "proteome" (the entire protein content). And, regrettably, one encounters "interactome," "phenome," and other "-omes" to describe component interactions, phenotypes, or just about anything else where the view is meant to be comprehensive or broad (Ge et al., 2003).

The two most common types of questions addressed by functional genomics are, "What portion of the genome is expressed in a particular cell or tissue (transcript cataloging)?" and, "How do the expression levels of genes change under different conditions (transcript or expression profiling)?" Since most organisms do not have the benefit of complete genome projects, the first question is typically asked in the context of gene discovery. The second question addresses gene function. Transcript levels generally correlate with physiological demand for their encoded products, though there are always exceptions. Thus, changes in transcript abundance can indicate which genes are becoming more or less important as a cell responds to a stimulus. Once regulated genes are identified, high-throughput

methods are also available to determine how they are regulated (reviewed in Ruan et al., 2004), though this type of analysis is still done mostly on a nongenomic scale.

Functional genomics is analogous to traditional molecular biology in the sense of gene discovery and characterization. The important difference is that the functional genomics perspective is holistic or global, in contrast to the reductionist approach traditionally taken by molecular biologists. For those used to concentrating on one or a few key genes at a time, functional genomics may appear unfocussed. Indeed most of the genes catalogued during a genomics study may seem to be peripherally germane to the issue at hand. However, genes often are important only in the context of their relationships to other genes, so the broader genomics perspective provides a more complete picture of how a cell functions. Functional genomics is, therefore, a powerful tool that can lead investigators to important genes that they might otherwise miss.

Before discussing various functional genomics applications, it is worthwhile to comment on equipment and processing. "High-throughput" refers to rapid, parallel processing of multiple samples, yielding data from hundreds or thousands of different clones. This type of processing is necessary because unreasonable amounts of time would be needed to generate the same information content by traditional molecular techniques. The repetitive nature of high-throughput processing is readily adapted to robots, which are more efficient and accurate than humans. Thus, there are robots for everything from arraying libraries into microtiter plates to preparing templates for polymerase chain reaction (PCR) and/or sequencing, and fabricating microarrays. Their purchasing costs are probably too high for most researchers to acquire them, so most "functional genomics" work is typically conducted at a company or core facility. Much of the bioinformatics work required to make sense of the genomics data is also automated. The result is that researchers actually do not "do" a lot of functional genomics—they contract the work to suitably equipped facilities. There are two obvious advantages to this. First, "specialized equipment" purchases are limited to small-scale items such as multi-well pipettors and disposable materials, so any typical molecular biology lab can readily adapt to functional genomics research. Second, it frees up time and resources for researchers to analyze the genomics results and confirm them. This "postgenomics" work is often necessary (see below, microarrays) and can require significant resources.

Transcript Cataloging: ESTs. Copy DNA (cDNA) is DNA produced by reverse transcription of RNA. These sequences are typically 1500–4000 nucleotides (nt) long. Expressed sequence tags (ESTs) are single-pass (i.e., unconfirmed by additional sequencing) cDNA sequences, usually generated from libraries. Most sequencing reads are typically in the 500–700 nt range, so ESTs are unlikely to cover the entire cDNA. They function as "tags" because they offer convenient preliminary identification of transcripts, as well as enough information to easily begin more thorough molecular characterization of corresponding full-length cDNAs

and/or genes, if desired. If the cDNA library is directionally cloned, investigators may choose to concentrate on either 5′ or 3′ ends to conserve resources. Larger scale projects, such as the effort to comprehensively mine the silkworm [*Bombyx mori* (L.) (Lepidoptera: Bombycidae)] transcriptome, may combine 5′ and 3′ reads and increase the probability of generating full-length sequences (Mita et al., 2003). EST databases are the only currently available approach to catalog gene representation in organisms with unsequenced genomes (i.e., nonmodel organisms). Serial analysis of gene expression (SAGE) and microarrays, typically used to monitor expression levels, can also be used to list expressed genes, but those methods need whole genome or cDNA/EST databases for support (see below). Functional genomic studies in nontarget organisms, thus, typically begin with EST database construction, followed by SAGE or microarray analysis to monitor transcript profiles.

The procedure is straightforward. A cDNA library in a plasmid vector (λ phage libraries need to be excised, or converted into plasmid form) is arrayed by randomly picking individual colonies into wells of a 96-well plate. Colony picking may seem arduous, particularly if several thousand clones are to be sequenced, but some judicious planning and a community-minded approach make manual colony-picking feasible and economical. Alternatively, robotic colony pickers may be contracted, if not purchased outright. Once the library is arrayed, the remaining steps: template DNA preparation, sequencing, and annotation, are mostly automated and a suitably equipped core facility is typically contracted to perform them.

Annotating the sequences usually involves assigning gene identities based on homology to known genes, typically using a BLASTX (Altschul et al., 1997) search of Swiss-Prot or Protein Information Resource (PIR) databases. There is a preference for sequencing 5′ ends rather than 3′ ends, because oligo-d(T)-primed cDNA libraries tend to under-represent 5′ untranslated regions (utrs). Thus, a sequencing read from the 5′ end is more likely to reveal coding region, whereas a read from the 3′ end may never emerge from the 3′ utr into useful sequence.

Sequencing redundancy refers to multiple reads of sequences for a gene. The frequency that a given cDNA occurs in a library generally reflects transcript abundance. On the one hand, redundancy is useful because it is a type of transcript profiling. In addition, it is sometimes possible to assemble contiguous ESTs into full length cDNAs. On the other hand, redundancy wastes resources when the objective is gene cataloging.

If the desire is to maximize the gene representation in a database, then steps are usually taken to minimize redundancy. One way to do this is to sequence small amounts (∼1000 cDNAs) from multiple libraries. The utility of this approach is illustrated by the *B. mori* EST project (Mita et al., 2003). Rather than construct a cDNA library from whole bodies, the project used 36 cDNA libraries from different tissues and/or developmental stages. Each library had between 17

and 49% library-specific clones, suggesting tissue-specific expression patterns (Kawasaki et al., 2003; Mita et al., 2003). A more traditional approach to minimize redundancy is normalizing and/or subtracting cDNAs prior to sequencing (Sasaki et al., 1994; Bonaldo et al., 1996; Carninci et al., 2000). Normalization equalizes the relative abundance of various cDNAs in a given sample, making it less likely to repeatedly encounter high-abundance cDNAs, and more likely to encounter low-abundance cDNAs (Ko, 1990). Subtraction is a process whereby tissue- or cell-specific mRNAs are separated from common mRNAs (e.g., from housekeeping genes) (Duguid and Dinauer, 1990). The *B. mori* multi-library approach will need to be supplemented with subtractive and normalizing techniques as more genes are represented by ESTs (Mita et al., 2003).

Alternatives to EST sequencing exist for transcript cataloging. These include SAGE (Velculescu et al., 1995) and massively parallel signature sequencing (MPSS) (Brenner et al., 2000). Both methods use short (14–20 nt) cDNA fragments as tags, rather than long fragments or entire cDNAs, and are much more efficient than EST sequencing. Data generated by these techniques are quantitative and immediately digitized, allowing easy direct comparison between samples. A drawback of early SAGE technology was some ambiguity in assigning tag identity. Even for those organisms with extensive genome data (e.g., the fruit fly, *Drosophila melanogaster* or the mosquito, *Anopheles gambiae*), there can still be ambiguity in mapping SAGE tags, as not all cDNAs have been fully sequenced (Pleasance et al., 2003). Recent advances, however, reduce ambiguity by increasing the size of the SAGE tags (Chen et al., 2002; Matsumura et al., 2003). SAGE is also more sensitive and can detect low-abundance sequences more efficiently than EST sequencing (Sun et al., 2004). MPSS, which is even more efficient than SAGE, has similar practical restrictions (a comprehensive, annotated database is required), and also requires specialized equipment currently offered exclusively by Lynx Biotechnologies (Brenner et al., 2000). Because of these limitations, EST sequencing remains the method of choice to catalog gene expression for nonmodel organisms.

If the goal is to identify only differentially regulated or highly expressed genes, then an extensive EST database becomes less important. Traditional methods such as subtractive techniques or differential display of mRNA (Liang and Pardee, 1992) can be used to enrich a pool of candidate sequences and tag them. Differential display has also been suggested as a possible tool to comprehensively mine the transcriptome for differentially-regulated sequences, much like EST sequencing or SAGE (Yang and Liang, 2004), though current applications typically identify a subset of differentially expressed genes. Resources can then be conserved by limiting sequencing to only those clones that pass a selection threshold. Similarly, SAGE and/or MPSS could be used to identify tags corresponding to highly expressed genes, provided that the tag can be used successfully for downstream cloning, e.g., via 3' and/or 5'RACE (rapid amplification of cDNA ends)-PCR.

It should be noted that microarrays can also be used for transcript cataloging, especially for organisms with fully sequenced genomes. In these cases, bioinformatics can predict every potentially transcribed portion of the genome, and a comprehensive microarray fabricated accordingly. When the array is queried using cDNA from a desired sample, those spots that do not return a signal would correspond to untranscribed genes. Indeed, this type of approach has been used to monitor expression profiles under different conditions (Wei et al., 2001), and even as a diagnostic technique (Cho et al., 2001; Whitfield et al., 2003).

Transcript Profiling: Microarrays. EST sequencing, SAGE, and MPSS can also be used to monitor expression levels, because transcript abundance is reflected in the population of isolated cDNAs. EST sequencing is not used to profile expression levels in practice because it is comparatively inefficient. SAGE and MPSS are both more efficient and more accurate, but require extensive supporting databases and, in the case of MPSS, specialized equipment. Microarrays also require some specialized equipment (printers for fabrication and scanners for analysis), but these are common in functional genomics core facilities. They also require a database to identify and track the genes represented on the microarray. Fortunately, the database is typically provided by a supporting EST project that is completed or at least well underway before the microarrays are fabricated. Thus, microarrays are probably the most popular technology for high-throughput expression profiling, particularly for nonmodel organisms. An alternative is to fabricate the array using uncharacterized cDNAs, hybridize it with a sample to identify target clones, and then return to the library to sequence the clones. This approach was recently used to good effect by Moon et al. (2004) to study the effects of protease inhibitors on cowpea weevils (see below).

Microarrays are glass microscope slides or specialized "gene chips" upon which single-stranded DNAs representing various genes have been fixed. They are analogous to northern dot blots, except that the DNA probes are unlabeled and fixed to a solid support, and the targets are a pool of labeled cDNAs synthesized from extracted RNA. This may be confusing to those accustomed to blotting RNA samples on membranes and hybridizing them with a labeled probe. Microarray experimenters, on the other hand, will discuss the unlabeled probes attached on their arrays, and the labeled cDNA samples they used to interrogate or query (hybridize to) them.

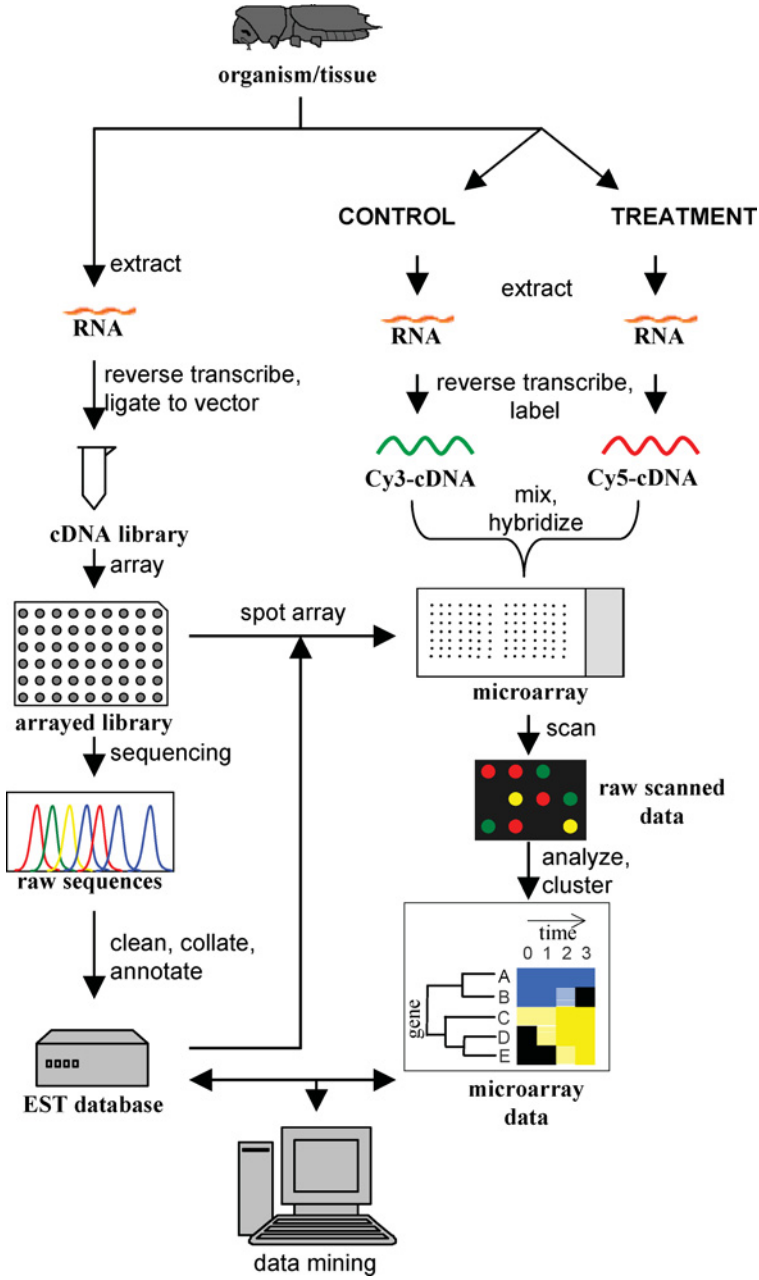
Despite the fact that microarrays are essentially multiple northern ("reverse-northern?") blots hybridized in parallel, it is misleading to interpret data from a microarray experiment in the context of individual genes. The objective is to understand how genes work together, and to learn about the interactive networks that they form. Thus, the more genes and the more conditions that are studied, the more we can learn about how the cell is functioning. This requires careful experimental design and statistical analysis (see bioinformatics, below) to pull the nuggets from the monumental volume of data that a microarray experiment

generates. Inevitably, small numbers of genes are identified through microarrays as being more useful predictors of physiological function, or as subjects for more conventional molecular analyses, but their identification as such is in the context of their expression relative to hundreds or thousands of other genes. Excellent reviews are available to guide investigators in this respect (Miller et al., 2002; Leung and Cavalieri, 2003).

Commercial arrays are available for those fortunate enough to work with model organisms (e.g., Affymatrix^{TM}). Researchers working on nonmodel organisms usually contract a core facility to fabricate custom arrays to suit their needs. The microarray solid support is typically a glass microscope slide coated with a surface chemistry that allows convenient probe attachment. Probe DNA is fixed in tiny, $\sim$100 μm diameter spots arranged in grids. Each probe spot on a microarray represents a gene, with replicate spots applied to monitor the quality of the array. The probe may be a cDNA fragment (library insert amplified by PCR) or a 20–50 nt synthetic oligonucleotide applied using a robotic dispensor (spotted array) (Hegde et al., 2000; Hughes et al., 2001). Alternatively, oligonucleotide probes may be synthesized *in situ* by photolithography (*in situ* array), a process currently offered commercially by Affymatrix^{TM} and NimbleGen^{TM}. As with EST sequencing, microarray construction requires specialized equipment, and core facilities or companies are commonly contracted to make them. Although the costs involved may appear high initially, the technology is easily scalable, and the amount of information recovered makes the investment worthwhile, particularly if researchers pool resources.

Aternatively, macroarrays can be synthesized easily in any molecular biology lab equipped with a dot blot apparatus. They are akin to microarrays, but use a larger surface, such as nylon membrane, and probe spots are large enough to be easily visualized. Although they tend to be more accurate than microarrays (Krebs et al., 2002), they are not nearly as comprehensive because they generally present fewer genes. They also are less flexible in the sense that control and experimental transcript levels must be measured sequentially, or on different macroarrays, whereas both samples can be assayed and compared directly on a single microarray slide.

Microarrays are often queried using a two dye format to learn how expression profiles change under different conditions (Figure 1). For example, RNA extracted from a control sample may be labeled with a fluor emitting at one wavelength, while RNA from the experimental sample is labeled with a different fluor emitting at a second wavelength. The fluors are typically cyanine 3 and cyanine 5 (Cy3 and Cy5, respectively), and a variety of methods are available to attach them to the RNA or cDNA (Randolph and Waggoner, 1997). The labeled samples are then mixed, hybridized with the probes on the chip, and washed much like a Southern or northern blot. The chips are read using specialized scanners (again, usually at a core facility) and analyzed with appropriate software. The ratio of signal strengths


organism/tissue
CONTROL
TREATMENT
extract
RNA
extract
RNA
RNA
reverse transcribe, ligate to vector
reverse transcribe, label
Cy3-cDNA
Cy5-cDNA
mix, hybridize
cDNA library
array
spot array
microarray
arrayed library
sequencing
scan
raw scanned data
raw sequences
analyze, cluster
clean, collate, annotate
time
0 1 2 3
gene
A
B
C
D
E
EST database
microarray data
data mining

for the two dyes indicates relative transcript abundance under the two conditions (reviewed in Hegde et al., 2000). If multiple conditions are assayed, the combined data can be extremely informative about the trend of transcript levels for different genes. Genes can be grouped according to their expression patterns (Eisen et al., 1998), and since coordinate gene regulation often implies meaningful interaction, significant insight into cell functions can be gained.

Experience from many labs shows that microarray data must be interpreted with caution. Relative expression levels are measured, not absolute transcript abundance. Also, the change in expression levels between conditions tends to be a qualitative indicator of trends within a cell (Kothapalli et al., 2002). Quantitative measurements are best left to more reliable techniques such as northern blotting (Taniguchi et al., 2001), semi-quantitative reverse-transcriptase (RT)-PCR, and quantitative real time RT-PCR (Rajeevan et al., 2001). At the very least, microarray results that are unconfirmed by any of these methods should be considered with low confidence (Haverty et al., 2004). Even after microarray results are validated, inferred pathways or responses to stimuli should still be checked biochemically, where possible. Microarray data are best viewed as a launch point for gene characterization, not a definitive indicator of function.

Published microarray experiments must conform to Minimal Information About a Microarray Experiment (MIAME) standards (Brazma et al., 2001). MIAME provides enough information about a given experiment to allow independent replication. It includes a description of the hybridization experiments, the design of the arrays (including information about each spot on the array), sample

FIG. 1. Flowchart of functional genomics experiments showing typical progression of methods, beginning from the whole organism. Gene cataloging is shown to the left. A cDNA library is constructed and arrayed into microtiter plates. Each clone is then sequenced, usually only once. The raw sequence data are then cleaned, organized, and annotated to create the EST database. Expression profiling is shown to the right. Microarrays are fabricated using the library clones and/or EST information. To compare expression levels under different conditions, RNA is extracted from both control and experimental (treatment) samples and converted to fluorescently-labeled first strand cDNA in separate reactions. In this example, the control cDNA is labeled with Cy3; the experimental cDNA is labeled with Cy5. The cDNAs are then mixed together, hybridized to the microarrays, and the resulting signals detected with a scanner. Color-coded images show spots representing genes that are expressed in the control sample, the treatment sample, or both. The lower right panel shows a typical output if the results of multiple experimental conditions are clustered using bioinformatics support. Each column represents an experimental condition (e.g., a time course), and each row a different gene. Colors (usually blue or yellow) indicate if relative expression from the experimental sample is higher or lower than the control sample (black indicates no difference in expression), and the intensity of the colors indicates the degree of difference.

identification and procedures used to extract and label RNA, hybridization and washing conditions, imaging specifications, and controls used to normalize signals. This information is typically deposited as supplementary online information in public repositories such as the European Bioinformatics Institute's ArrayExpress (Brazma et al., 2003), or the Gene Expression Omnibus (GEO) maintained by the National Center for Biotechnology Information (NCBI) in the USA (Edgar et al., 2002).

Bioinformatics. Computer-assisted analysis is imperative for maintaining, sorting, and analyzing functional genomics data. Fortunately, bioinformatics can be the least expensive portion of a functional genomics study because a variety of freeware is available to academic scientists (Table 1). Products are also available for purchase from bioinformatics companies. These packages tend to be more user-friendly, and offer more integrated abilities than freeware, but they can be expensive.

Much of the analysis is automatic, and this is necessitated by the volume of information at task. For example, the EST Analysis Pipeline (ESTAP) from the Virginia Bioinformatics Institute (Mao et al., 2003), automatically trims and cleanses raw data from a sequencer, clusters redundant or contiguous sequences, and annotates the ESTs based on BLASTX searches at NCBI. In my lab, our EST sequences began accumulating before our bioinformatics support was ready, so we began annotating them one by one. It took nearly a month. When ESTAP was ready, it did the same work more accurately and comprehensively in a few hours (Eigenheer, 2002). Since so much of functional genomics is done at core facilities and/or by computers, researchers can use their time to confirm/verify all the data.

By mid-2004, there were over 765,000 ESTs from the Insecta listed in dbEST (Information, 2004), most of these from large-scale efforts (Table 1). This compares favorably with the $\sim$1.6 million confirmed insect sequences reported in the GenBank nonredundant (nr) database. Annotating new ESTs continues to become increasingly easy and accurate as more sequences accumulate. However, new projects routinely reveal $\sim$50% unknown cDNAs, meaning a sizable fraction of genes still have uncharacterized functions and we still have a long way to go to understand them.

Microarrays in particular require bioinformatics support. Consider that a typical array may have at least duplicate or triplicate spots for hundreds or thousands of genes. Recovering information from a single spot requires differentiating the signal from the background, and averaging the signal strength across the entire spot, not to mention averaging values for replicates and normalizing values across multiple arrays and background values. Beyond simply recovering the information from a chip, sorting and analyzing the data for trends, and presenting this information in a readily understandable format requires considerable computer assistance.

TABLE 1. SOME AVAILABLE ONLINE BIOINFORMATICS RESOURCES

	Name	URL
EST databases		
Various organisms	TIGR gene indices	http://www.tigr.org/tdb/tgi/
Apis mellifera	Honey Bee Brain EST Project	http://www.biotec.uiuc.edu/bee/honeybee_project.
Aedes and other mosquitoes	Mosquito Genomics WWW Server	http://klab.agsci.colostate.edu/
An. gambiae	AnoBase	http://www.anobase.org/
B. mori	SilkBase	http://samia.ab.a.utokyo.ac.jp/silkbase/
Other Lepidoptera	International Lepidoptera genome project	http://www.ab.a.utokyo.ac.jp/lepgenome/index.
Drosophila melanogaster	FlyBase	http://flybase.bio.indiana.edu/
Drosophila pseudoobscura	Drosophila Genome Project	http://www.hgsc.bcm.tmc.edu/projects/drosophila/
Other Drosophilidae	Berkeley Drosophila genome project	http://www.fruitfly.org/comparative/index.
Glossina morsitans morsitans	*Glossina morsitans* EST sequencing	http://www.sanger.ac.uk/Projects/G_morsitans/
Tribolium castaneum	BeetleBase	http://www.bioinformatics.ksu.edu/BeetleBase/index.
Ips pini and other beetles	BarkBeetleBase	http://bioinformatics.unr.edu/beetle/
GenBank ESTs	dbEST	http://www.ncbi.nlm.nih.gov/dbEST/index.
EST Freeware		
University of Edinburgh	Edinburgh EST pipeline	http://envgen.nox.ac.uk/est.html
Virginia Bioinformatics Institute	ESTAP	http://staff.vbi.vt.edu/estap/
Microarray databases		
MIAME/MAGE resources	Microarray Gene Expression Data Society	http://www.mged.org/index.html
ArrayExpress	ArrayExpress	http://www.ebi.ac.uk/arrayexpress/index.
Gene Expression Omnibus	NCBI-GEO	http://www.ncbi.nlm.nih.gov/geo/
Microarray freeware		
M. Eisen, Stanford University	ScanAlyze, Cluster, SAM, etc.	http://staff.vbi.vt.edu/estap/
M. Sturn, Graz U. Technology	Genesis	http://genome.tugraz.at/Software/GenesisCenter.
National Center for Genome Research	GeneX	http://www.ncgr.org/research/genex/
The Institute for Genome Research	MIDAS, Spotfinder, MADAM, etc	http://www.tigr.org/software

Useful bioinformatic analysis assumes high quality data; if the experiment is poorly designed or executed, no computer on earth will pull out something meaningful. The sheer volume of information often means that researchers risk finding desirable, though not necessarily relevant or significant correlations to suit their biases (Miller et al., 2002). False positives and negatives can be attenuated through careful experimental design and statistical analysis of experimental data, minimizing the risk of arriving at unsubstantiated interpretations (reviewed in Nadon and Shoemaker, 2002). Fortunately, a great deal of help for experimental design and analysis is available from a thriving community of microarray researchers in the form of regular seminars, training sessions, and online-bulletin boards (e.g., the microarray listserver at http://www.gene-chips.com/gene-arrays.html).

FUNCTIONAL GENOMICS AND INSECT PHEROMONE BIOSYNTHESIS

ESTs have been used to identify pheromone-biosynthetic genes in *B. mori*. Bombykol biosynthesis requires a fatty acyl-CoA desaturase, but such desaturases are typically members of multigene families, so, without having to clone and study the expression patterns of each, how could researchers find the one that is required for pheromone biosynthesis? In this case, a convenient EST database from pheromone glands was part of a larger effort to catalog the transcriptome of *B. mori* (Mita et al., 2003), and the researchers were identifying genes they strongly expected to exist. Of the desaturase enzymes identified through ESTs, the vast majority represented a Δ-9 desaturase, which was confirmed to be pheromone-gland specific (Yoshiga et al., 2000). Similarly, researchers isolating pheromone-biosynthetic fatty acyl-desaturases from *Dr. melanogaster* relied on established EST databases to assist with their cloning (reviewed in Jallon and Wicker-Thomas, 2003).

A pioneering effort to use functional genomics to understand pheromone biosynthesis involves studies of ipsdienol biosynthesis by the bark beetle, *Ips pini* (Say) (Coleoptera; Scolytidae) (Eigenheer et al., 2003). Male *I. pini* synthesize ipsdienol *de novo* via the mevalonate pathway as an aggregation pheromone component (Seybold et al., 1995b). Synthesis occurs in anterior midgut cells (Hall et al., 2002), and is regulated by juvenile hormone III (JH III) (Tillman et al., 1998; reviewed in Seybold and Tittiger, 2003). HMG-CoA reductase gene (*HMG-R*) expression and enzyme activity levels are stimulated by JH III (Tillman et al., 2004), consistent with *HMG-R*'s established role as an important mevalonate pathway regulator (Goldstein and Brown, 1990). *HMG-R*'s robust, cell-specific response to JH III suggested the system could be a useful new tool to study the mechanism of JH action (Tittiger, 2003). Functional genomics was the best way to uncover potential components without biasing experiments with precedents from other model systems (e.g., steroid hormone receptors, *etc*.). The project would

have the added benefit of answering some other relevant questions: How is the mevalonate pathway regulated? How is carbon shunted away from the normal long-chain terpenoid products into the relatively short (and unusual) monoterpenoid pheromone components? What other enzymes are involved in ipsdienol biosynthesis? Finally, some of the ESTs could be expected to represent *I. pini*-specific genes. Though not necessarily pheromone-related, these genes could be targets for future directed control strategies if ways to disrupt them (e.g., RNAi or specific inhibitors) are developed. These considerations provided the motive for a small-scale EST project and microarray-based analysis of *I. pini* midguts (Tittiger, 2003).

In total, 1671 useful ESTs were obtained from a directionally-cloned cDNA library of mRNA isolated from pheromone-biosynthetic midguts (Eigenheer et al., 2003). From the beginning, redundancy scores were unusually high, and despite efforts to increase sequencing efficiency by prescreening, sequencing was terminated after only 30 plates due to unacceptably high sequencing redundancy. The ESTs were sorted into clusters or singlets, resulting in 574 tentative unique genes (TUGs). Two clusters, representing genes or gene families provisionally named *IPG001B01* and *IPG001D12*, accounted for 35% of all the sequenced ESTs and contributed most of the redundancy. Other highly-represented sequences included catabolic enzymes, as would be expected in digestive tissues (Eigenheer et al., 2003). This provides a cautionary note: the database would be more diverse had a normalized library been used to reduce the frequency of highly expressed cDNAs.

The ability of the adult *I. pini* EST database to provide information about tissue function was tested by comparing it with midgut ESTs from larval *B. mori* (Eigenheer et al., 2003). When TUGs from both insects were sorted into functional categories, the fractions representing energy, cell growth, protein trafficking, structure, signaling, and detoxification were roughly similar for each insect, whereas fractions for metabolism, translation, transport, and unknown were quite different (Figure 2). The metabolism category is especially interesting since midguts from both species have similar digestive functions, and indeed, most identifiable orthologs are digestive enzymes. However, the frequency of lipase cDNAs reflects dietary differences between the insects: larval silkworms feed on mulberry leaves, which have a much higher neutral lipid content than the pine phloem fed on by adult bark beetles. Most significantly, mevalonate pathway genes are represented in *I. pini*, but not *B. mori*, TUGs, consistent with the additional substantial metabolic load of synthesizing ipsdienol by adult male *I. pini* midguts. The higher overall fraction of metabolic TUGs in *I. pini* midguts may also reflect this difference (Eigenheer et al., 2003).

The "unknown" TUG category is also interesting as a source of potential species-specific clones. Although this fraction is markedly smaller in *I. pini* compared to *B. mori*, the difference is due largely to the fact that almost 3/4 of the *I. pini* unknown ESTs correspond to *IPG001B01* or *IPG001D12*. These two sequences

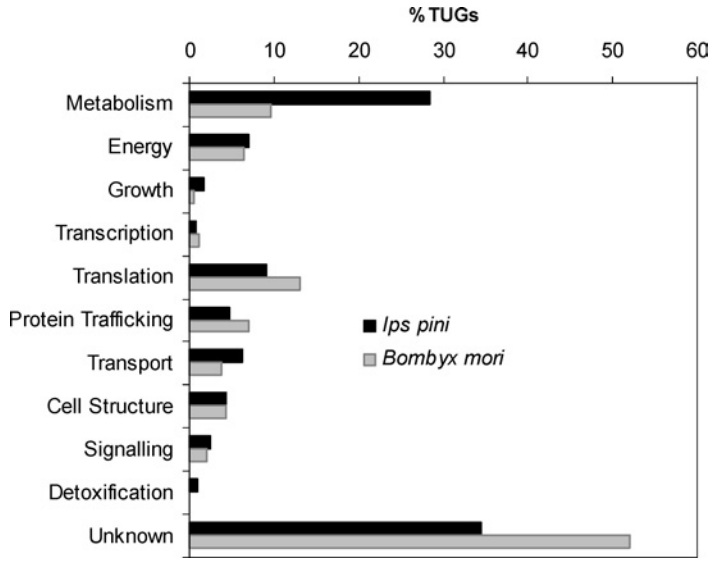


FIG. 2. *Bombyx mori* and *Ips pini* EST representation in functional categories. BLASTX searches were used to assign putative identities to tentative unique genes (TUGs) from *B. mori* and *I. pini*. Figure reproduced from Eigenheer et al. (2003) with permission.

are not represented in GenBank nr, other beetle EST projects (Girard and Jouanin, 1999; Theodorides et al., 2002; Pedra et al., 2003), or insect databases, including the *Tribolium castaneum* genome project. Apparent homologs are present in some *Dendroctonus* spp. bark beetles (Tittiger, unpublished observations), suggesting that they are unique at least to the Scolytidae.

The seven mevalonate pathway genes recovered from the EST project allowed investigation into how the pathway was regulated. Since female *I. pini* do not synthesize ipsdienol (Seybold et al., 1995a), the expression of mevalonate pathway genes was not expected to increase in female midguts after feeding. Quantitative (real time) PCR analysis of known *I. pini* mevalonate pathway genes confirmed that feeding coordinately stimulates the pathway in male midguts (Keeling et al., 2004a). Surprisingly, early steps in the pathway (up to isomerization of isoprenyl diphosphate to dimethylallyl diphosphate) were also stimulated in female midguts, while later steps were not. Even though expression levels of these genes in females were consistently lower than in males, the relative responses to feeding were almost the same. A second surprise was that geranyl diphosphate synthase (GPPS), which regulates the step at which carbon is shunted away from the main mevalonate pathway into monoterpenoid products was not strongly responsive to feeding in males (reviewed in Tittiger, 2003). Instead, basal transcript

levels for all studied mevalonate pathway genes, and *GPPS* in particular, were significantly higher in males compared to females. These data imply that pheromone biosynthesis in *I. pini* is much more complex than originally anticipated. Some pheromone-biosynthetic genes are strongly induced by feeding, while others are already highly expressed, probably in response to developmental or environmental cues (Keeling et al., 2004a).

The effect of topical JH III application and feeding on gene expression in *I. pini* midguts has been studied using microarrays prepared from the ESTs (Keeling et al., 2004b). The results confirm that most mevalonate pathway genes are coordinately up-regulated by JH III in male midguts. In addition, nearly a quarter (94 of 384) of the genes represented on the chip significantly elevated or reduced expression levels in male midguts following topical JH III treatment. While some of these are clearly pheromone-biosynthetic (e.g., mevalonate pathway genes), others are of unknown function (Keeling et al., 2004b). The potential roles of some of these—from JH III signal transduction and/or sub-cellular reorganization, to pheromone production, transport, release—are currently under investigation.

FUNCTIONAL GENOMICS AND INSECT PHEROMONE/ODOR RECEPTION

The molecular basis for reception of pheromones and other odors includes various associated binding proteins, receptors, degrading enzymes, and signal transduction pathways (reviewed in Jacquin-Joly and Merlin, 2004). Since the characterization of the first pheromone-binding protein (PBP) over 20 years ago (Vogt and Riddiford, 1981), numerous examples of PBPs and odorant-binding proteins (OBPs) have emerged, largely with the help of genome projects (reviewed in Vogt, 2003). As data accumulated, it became evident that OBPs have highly divergent sequences, making their cloning refractory to traditional homology based molecular techniques. EST-based cloning would theoretically avoid this limitation and identify multiple components of the pheromone-sensing apparatus.

An example of a small-scale EST project utilized to identify new OBPs is a survey of tobacco hornworm, *Manduca sexta* (L.) (Lepidoptera: Sphingidae), male antennal cDNAs (Robertson et al., 1999). In this study, 375 cDNAs synthesized from male antennal mRNA were analyzed, revealing four new OBPs. While this illustrates the utility of limited EST projects, many interesting clones, particularly those hypothesized to be involved in signal transduction, were not recovered, presumably because of their low transcript abundance (Robertson, et al., 1999). More complete genome analyses (e.g., of *Dr. melanogaster* and *An. gambiae*), of course, lead to more complete surveys (Biessmann et al., 2002; Vogt et al., 2002) and a more comprehensive view of odorant sensing components that should greatly help our understanding of pheromone reception.

Microarrays have been used to study pheromone reception in the honey bee, *Apis mellifera* (Hymenoptera: Apidae) (Grozinger et al., 2003; Whitfield et al., 2003). It is worth noting the EST database supporting the microarray work. To rectify a scarcity of known bee sequences, over 15,300 ESTs, representing over 8900 genes, were recovered from adult male *Ap. mellifera* brains (Whitfield et al., 2002). The study is wonderfully thorough. An exhaustive survey (over 20,000 sequenced clones) of a subtracted/normalized brain-specific library yielded a database representing an estimated 50% of the bee's transcriptome from a single tissue source (Whitfield et al., 2002). Extensive controls and checks to monitor redundancy minimized annotation mistakes and ensured an accurate product. This work serves as a rigorous benchmark for others to follow when designing their own EST projects.

The database and its associated microarray of 7000 putative different genes is not particularly helpful to learn about how odors are received and processed, but it does make possible the study of how odorants affect gene expression in the brain and, by implication, behavior. Before the database was complete, and even before the cDNA library was normalized for sequencing, custom microarrays of unannotated clones were used to identify gene expression (Kucharski and Maleszka, 2002) specific for newly emerged adults, experienced foragers, or adults treated with caffeine. The identity of differentially regulated genes was established through sequence analysis only after they were revealed by microarray hybridization (Kucharski and Maleszka, 2002). Alternatively, a statistical analysis of different, annotated microarrays identified at least 50 cDNAs (out of over 2700 significantly regulated genes) whose expression patterns accurately predicted the behavioral status of individual bees (foragers vs. nurses) (Whitfield et al., 2003). Interestingly, many of the significantly regulated genes in this and a following study (Grozinger et al., 2003) had $<20\%$ change in expression levels, reinforcing the paradigm that biologically significant changes may be affected by less than a two-fold change in expression. In fact, some genes with the strongest fold-change in expression levels in pooled samples were not as consistently regulated as the less-strongly responding genes (Whitfield et al., 2003).

To date, the only publication of a genomics-based analysis of pheromone reception in insects describes the effects of queen mandibular pheromone (QMP) on gene expression in adult *Ap. mellifera* brains (Grozinger et al., 2003). QMP is a multi-component pheromone important in regulating worker development and behavior (Plettner et al., 1996). Microarray analysis of $\sim$7600 genes revealed $\sim$1200 that were significantly up-regulated, and $\sim$1300 that were significantly down-regulated in QMP-exposed workers. As indicated above, these involved changes in expression much smaller than two-fold. Another interesting result was that transcription factors were more highly regulated than other functional groups, such as phosphatases, kinases, receptors, *etc*. This suggests that pheromones can trigger developmental programs by activating one or a few transcription factors,

which in turn would regulate downstream genes. Some of the *Ap. mellifera* transcription factors responded transiently, implying that they were required only to initiate a cascade; whereas the *Drosophila Kruppel* homolog (*Kr-h1*) had a more long-term response, suggesting a continuous role in facilitating a sustained change in physiology and behavior (Grozinger et al., 2003).

FUNCTIONAL GENOMICS AND PLANT-INSECT INTERACTIONS

It is clear that the genomes of phytophagous insects and their hosts interact with each other through the organisms carrying them. Functional genomics has been applied to this interaction from the plant perspective with studies on the response of tobacco, *Nicotiana attenuate*, genes to herbivory by *M. sexta* (Halitschke et al., 2003; Hui et al., 2003). In these studies, plant genes that responded to *M. sexta* were first identified by differential display PCR, and then spotted onto microarrays for further study. The arrays were, thus, on a small scale, representing between 115 and 225 genes. They were used to determine which genes responded to wounding as opposed to insect secretions, as well as to various components in the insect secretions. Interestingly, over half of the plant genes that were induced by *M. sexta* regurgitant could also be induced by the fatty acid–amino acid conjugates in the regurgitant, suggesting that plants may recognize their predators via the composition of their oral secretions (Halitschke et al., 2003)

From the insect perspective, the response of the cowpea weevil, *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae), to defenses produced by cowpeas, *Vigna unguiculata*, has similarly been investigated using microarrays (Moon et al., 2004). Cowpeas produce a cysteine protease inhibitor, soyacystatin N (scN) that inhibits digestion in the weevils. In this study, a subtractive technique was used to enrich a cDNA library with clones representing genes that were either induced or suppressed in the larval gut by scN. A microarray was then fabricated using these cDNAs, and queried with samples prepared from beetles that were differentially adapted to scN (Moon et al., 2004). Of 1920 cDNAs on the array, 151 appeared induced or suppressed by scN. Among those induced were proteases, carbohydrate digestive enzymes, and microbial defense and detoxification genes.

It is worthwhile noting the strategies of the above two studies. In both cases, steps were taken to enrich a cDNA pool to represent differentially regulated genes before microarrays were used. Microarrays were more of a confirmatory tool, particularly for the studies in tobacco plants (Halitschke et al., 2003). An advantage of this approach is that sequencing is limited to cDNAs representing responsive genes, which may conserve resources. Indeed, although *C. maculatus* larval midgut ESTs were presumably available (Pedra et al., 2003), sequencing was performed only after the microarray experiments identified differentially-regulated genes (Moon et al., 2004). A disadvantage is that the perspective is

limited to strongly responsive genes, since these are most likely to be retained during the prescreening steps. Other physiologically important genes may have been missed if their shifts in expression were more subtle.

FUTURE DIRECTIONS

Applications of genomics technology to entomology was recently both celebrated for the volume and quality of data generated, and lamented for the pace at which the approach was becoming accepted as *de rigeur* (Tautz, 2002). The entry of genomics into routine molecular research is perhaps best illustrated by the fact that the United States Department of Agriculture has earmarked funding for genomics projects in agriculturally important insects and mites. Functional genomics is being applied more and more in every aspect of life science research, including research in ecology and evolution (reviewed in Feder and Mitchell-Olds, 2003). Chemical ecologists have been perhaps more conservative than others in embracing molecular technologies, but they are probably more used to handling quantitative data than molecular biologists. Many statistical tools normally used by chemical ecologists could probably be adapted to analyzing functional genomic data. So why wait? More and more complete genomes are available (*Drosophila melanogaster*, *An. gambiae*) and others are either in progress (*B. mori*, *Heliothis virescens*, *T. castaneum*, *Ap. mellifera*) or planned (*Drosophila pseudoobscura*). Small-scale EST projects (i.e., a few hundred to a thousand sequences) are becoming more common as tools for gene discovery (Theodorides et al., 2002; Eigenheer et al., 2003; Pedra et al., 2003), and concerted efforts are being made to generate ESTs for many insects. For example, several thousands of ESTs are planned for various species as part of an effort to use comparative genomics to understand Lepidoptera (Feyereisen, 2002).

Microarray analyses still are mostly limited to the most common model organisms (*Drosophila, Apis,* and *Bombyx*), but more can be expected from species that present interesting research questions. It is also becoming evident that heterologous hybridizations—using samples from one species to query cDNA microarrays from another—can provide useful data, provided that the evolutionary distance is reasonably close (Renn et al., 2004; Rise et al., 2004). This ability greatly reduces the costs involved with intra-specific studies.

For bark beetle studies, a second generation *I. pini* microarray containing all 574 identified TUGs is now available (Keeling et al., personal communication). An EST database is being constructed from a normalized midgut library for the mountain pine beetle, *Dendroctonus ponderosae* (Coleoptera: Scolytidae), and associated microarrays should be soon available (Tittiger et al., unpublished data). Inter- and intra-specific bark beetle nucleotide sequence similarities are high enough that heterologous screening should be possible. For example, the *I. pini* microarrays

are currently being interrogated with samples from the pinyon ips, *Ips confusus* (M. Ginzel and G. Blomquist, personal communication), and future *De. ponderosae* arrays can be queried with samples from *Dendroctonus jeffreyi* and *Dendroctonus frontalis*. Comparisons between and across the various databases will provide exciting new information on how pheromone systems and their regulatory strategies have evolved. They should also identify new targets for pest control.

Hot on the heels of functional genomics comes proteomics: the study of how proteins within a cell interact. In this approach, crude cellular extracts are separated by two-dimensional polyacrylamide gel electrophoresis. Comparison of gels run with different samples identifies changes (appearance, disappearance, or shifted intensity) in particular proteins (Yarmush and Jayaraman, 2002). Like expression profiling, protein profiling does not give information about the activities of functional, correctly localized proteins or their metabolites. However, proteins are one step further from the genes, and one step closer to function, so in a sense, proteomics is more informative than functional genomics. Because proteins are identified typically through mass spectrometry of peptide fragments, proteomics requires an annotated EST database for support.

Metabolomics is essentially the study of the complete biochemical phenotype of a cell, and, thus, the metabolic end-products of the transcriptome and proteome (Sumner et al., 2003). While it avoids the vagueness of transcriptome and proteome analysis (a sizable fraction of cDNAs and proteins are unknown), current technologies cannot comprehensively monitor a tissue's every metabolite (reviewed in Phelps et al., 2002). However, researchers can focus their analyses on certain types of metabolites and monitor their behavior. For chemical ecologists, metabolomics approaches could return great benefits by tracing the fate of semiochemicals after they enter a sensillum, or by monitoring a pheromone-biosynthetic pathway.

Finally, the integration of any or all of these technologies yields a holistic view of cell function, and this is often called systems biology (reviewed in Ge et al., 2003). The ability to selectively perturb cell metabolism used to be the exclusive domain of targeted genetic knockouts. This technique once was limited to selected model organisms with highly specialized genetic and molecular tools, but interference RNA (RNAi) techniques that achieve similar effects are now developing rapidly and becoming more widespread. Selective knockouts or knockdowns can theoretically be done for any gene in any organism (Nishikawa and Natori, 2001). With access to these tools, chemical ecologists have unprecedented opportunity to learn more about the biochemistry and ecological ramifications of their favorite systems more quickly and comprehensively than ever.

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REVIEW ARTICLE

INSECT OLFACTORY RECEPTORS: CONTRIBUTIONS OF MOLECULAR BIOLOGY TO CHEMICAL ECOLOGY

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Abstract—Our understanding of the molecular basis of chemical signal recognition in insects has been greatly expanded by the recent discovery of olfactory receptors (Ors). Since the discovery of the complete repertoire of *Drosophila melanogaster* Ors, candidate Ors have been identified from at least 12 insect species from four orders (Coleoptera, Lepidoptera, Diptera, and Hymenoptera), including species of economic or medical importance. Although all Ors share the same G-protein coupled receptor structure with seven transmembrane domains, they present poor sequence homologies within and between species, and have been identified mainly through genomic data analyses. To date, *D. melanogaster* remains the only insect species where Ors have been extensively studied, from expression pattern establishment to functional investigations. These studies have confirmed several observations made in vertebrates: one Or type is selectively expressed in a subtype of olfactory receptor neurons, and one olfactory neuron expresses only one type of Or. In addition, all olfactory neurons expressing one Or type converge to the same glomerulus in the antennal lobe. The olfactory mechanism, thus, appears to be conserved between insects and vertebrates. Although Or functional studies are in their initial stages in insects (mainly *Drosophila*), insects appear to be good models to establish fundamental concepts of olfaction with the development of powerful genetic, imaging, and behavioral tools. This new field of study will greatly contribute to the understanding of insect chemical communication mechanisms, particularly with agricultural pests and disease vectors, and could result in future strategies to reduce their negative effects.

Key Words—Insects, olfaction, molecular biology, olfactory receptors, G-protein coupled receptor, SNMP, CD36, guanylyl cyclase, arrestin, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Anopheles gambiae*.

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INTRODUCTION

Animals have developed highly specialized sensory organs to detect physiochemical characteristics in their environment. These signals are used in many aspects of their lives, including behavior, physiology, and metabolism. Vertebrates and insects share a common design in their olfactory system, with a similar cellular and molecular organization. Insects present particularly good models for basic studies on the sense of smell because they offer the possibility of using genetic engineering to alter their olfactory system, followed by fairly simple behavioral assessments. Moreover, among insects are found economically important agricultural pests and disease vectors, for which olfaction underlies behaviors that are critical for mate or host recognition and selection. Numerous studies have been conducted on chemical ecology of insects since the discovery of the first pheromone in the silkmoth, *Bombyx mori* (Butenandt et al., 1959), including identification and structural determination of chemical signals, and the corresponding electrophysiological and behavioral responses. Until 1980, however, little was known about the molecular elements involved in odorant detection at the level of the antennae. The development of biochemical and molecular studies then increased our knowledge of the molecular basis of chemical communication. In particular, our understanding of chemical senses was greatly expanded by the discovery of olfactory receptor (Or) gene families, first in the vertebrate *Rattus norvegicus* (Buck and Axel, 1991), and then in the nematode *Caenorhabditis elegans* (Troemel et al., 1995). The recent identification of Ors in insects is now providing new opportunities to understand the molecular basis of the general framework of olfaction.

This review focuses on the present knowledge of insect Ors, with some references to vertebrate Ors, in order to emphasize the striking evolutionary convergence towards a conserved organization of signaling pathways in olfactory systems. Olfactory receptors are implicated in one step of the olfactory cascade of events, which consist of combinatorial systems from stereochemical recognition to the generation of an odor code in the brain. Thus, Ors belong to a succession of interactions that, in concert, lead to the animal response. From the discovery of Ors to their first functional studies, we show what the molecular aspects of odor detection may bring to the understanding of chemical communication. Indeed, the discovery of Ors is one of the best examples of the application of the powerful tools of genetics and genomics to chemical ecology. Finally, we discuss the possible practical applications of this new field of study.

GENERAL OLFACTORY PROCESS

Insect chemical odorant messages are translated into neuronal electrical activities through specialized organs, principally the antennae, and processed by

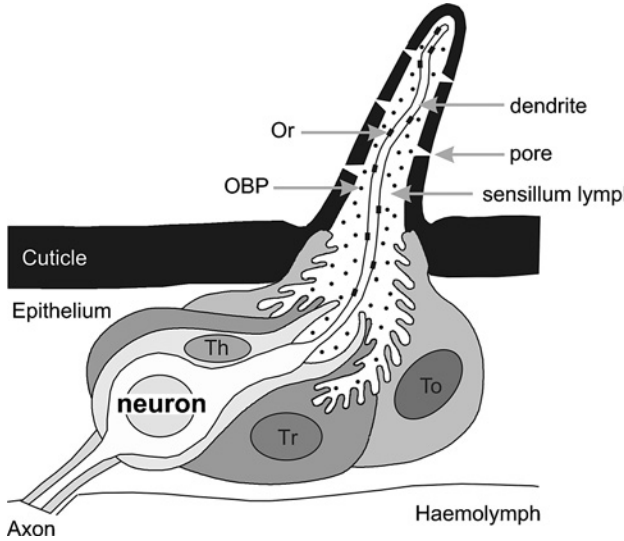

FIG. 1. General organization of an insect olfactory sensillum. Olfactory receptor neurons are bipolar cells: surrounded by accessory cells (To: tormogen, Th: thecogen, and Tr: trichogen cells). The dendrite is bathed in the sensillum lymph. Odorant binding proteins (OBPs) are soluble proteins present in the lymph, whereas olfactory receptors (Ors) are membrane proteins associated with the dendrite (modified from Steinbrecht et al., 1992).

brain centers to elicit behavioral–physiological responses. The antennae are composed of morphofunctional units, the sensilla, which contain one or several bipolar olfactory receptor neurons (ORNs) (Figure 1). The ORNs are specialized at the dendritic end for chemical detection and at the axonal end for neuronal signaling. These specialized sensory neurons transduce the chemical signal into an electrical response and bring the olfactory information from the periphery to the antennal lobes (ALs), which are the first relay stations in the brain (Hansson, 1995; Hildebrand, 1996; Mustaparta, 1996). The dendrites of olfactory neurons are bathed in an aqueous sensillar lymph that protects them from dehydration.

The incoming odor experiences several different extracellular steps, called the perireceptor events or early olfactory processing. These events range from odorant capture to the activation of a neuronal receptor. The generation of the corresponding electrical message, i.e., the intracellular events, is referred to as signal transduction *sensu stricto*. Three protein constituents are involved in the perireceptor events, the odorant-binding proteins (OBPs), the odor-degrading enzymes (ODEs), and the Ors of the sensory neurons (Vogt and Riddiford, 1981). After entering the sensillum through cuticular pores, the odorant molecule has to cross the aqueous lymph to reach the dendrite of the olfactory neuron. As most

odorant molecules are highly volatile and relatively hydrophobic compounds, they are bound by OBPs as they cross the lymph. More than functional carriers, these OBPs appear to act in solubilization and in first selection of olfactory information (reviewed in Leal, 2003). When the odorant–OBP complex arrives at the dendritic membrane, the odor reaches the transmembrane receptor, although it is not clearly understood if the complex dissociates near the receptor, which then binds to the odor alone, or if the complex itself docks with the receptor. Ors then play a dual role. First they allow discrimination among different odorants, as only cells possessing a suitable receptor type will respond to the odorant. Second, they transfer the chemical message from the extracellular to the intracellular face of the membrane upon binding with the ligand (or agonist). This phenomenon elicits a cascade of events leading to the nervous activity. Electrical signals are conveyed onto higher brain centers where they are integrated and contribute to elicit appropriate behavioral responses. Signal termination also plays a critical role in the olfactory process. It involves ODEs, soluble extracellular as well as intracellular membrane-bound and cytosolic enzymes, which are supposed to participate in ligand degradation after their interaction with receptors (Vogt, 2003).

EXPLORATORY INVESTIGATIONS OF OLFACTORY RECEPTORS IN INSECTS

Since the early 1980s, many investigations have been conducted to identify gene products that play a role in odor detection. Involvement of receptor molecules was suspected based on structure activity studies (e.g., Kafka and Neuwirth, 1975; Kikuchi, 1975), and their proteinaceous nature was suggested because chemicals that disrupt protein structure also disrupt odor response pathways (e.g., Villet, 1974; Frazier and Heitz, 1975). Moreover, other studies provided evidence for a second messenger pathway (e.g., Villet, 1978; Wieczorek and Schweikl, 1985) and a G-protein-mediated reaction cascade (Breer et al., 1994) elicited in olfactory sensory cells upon odorant stimulation. Functional approaches were developed to search for putative Ors in insect antennae. These studies led to the discovery of diverse proteins involved in the mechanism of insect olfaction. Vogt and Riddiford (1981) developed a pheromone binding test that allowed identification of proteins from a sensillar extract of the moth, *Antheraea polyphemus*. Using this assay, the first pheromone-binding protein (PBP) and pheromone-degrading esterase (PDE) were discovered. However, no Or was revealed using this assay system (Vogt and Riddiford, 1981).

DISCOVERY OF THE FIRST CANDIDATE Ors IN VERTEBRATES

The first Ors were discovered in the rat olfactory epithelium (Buck and Axel, 1991). These workers used an innovative approach that assumed that the Ors

would be members of the G-protein coupled receptor (GPCR) super family and encoded by genes expressed only in olfactory tissues. Indeed, GPCRs are involved in a variety of cellular processes, such as hormonal regulation, neurotransmission, and photoreception. Using degenerate oligonucleotide primers designed to anneal with conserved regions in the transmembrane domains (TMs) of the GPCR family, Buck and Axel (1991) used polymerase chain reaction (PCR) to amplify and identify complementary DNAs (cDNAs) encoding *Or* genes. Their homology-based approaches proved to be successful and led to the identification of several hundred *Or* genes selectively expressed in olfactory neurons.

Since then, *Or* genes have been identified from a variety of vertebrates, including humans (Ben-Arie et al., 1994), fish (Ngai et al., 1993), and birds (Nef et al., 1996), revealing strong conservation across the Chordata (Mombaerts, 1999). By analyzing genomic DNA sequences from the invertebrate nematode *C. elegans*, Troemel et al. (1995) identified a large family of genes that were expressed in chemosensory neurons and that encoded receptors with seven TMs. Robertson (2001) further characterized this gene family. These genes, named *sr* (serpentine receptor), are extremely divergent within *C. elegans*, and present poor primary sequence identity with vertebrate *Ors*, reflecting an apparently independent evolutionary origin. *Or* number varies widely among species, from approximatly 1000 genes in mammals and 800 in *C. elegans* to approximately 100 in fish and birds (Mombaerts, 1999).

Although thousands of putative Ors have been described in many species, up to now, few studies have reported that the corresponding proteins bind odorants (see "Or Functional Studies").

GENERAL PROPERTIES OF GPCRs

G-protein coupled receptors comprise a large membrane protein family whose members share many common features. They belong to a three-part complex that involves a membrane receptor for external signal reception, a heterotrimeric transducer (G protein), and one of several effector enzymes (e.g., phospholipase C or adenylyl cyclase) leading to the synthesis of second messengers such as inositol 1,4,5-triphosphate ($InsP_3$) or cyclic AMP (cAMP) (Krieger and Breer, 1999; Breer, 2003). These receptors have seven transmembrane regions (TMs) with the N-terminus of the protein situated outside the cell, defining an external domain, and the C-terminus situated inside the cell cytoplasm (Figure 2A) (Buck and Axel, 1991). The seven TMs are characterized by the presence of highly hydrophobic amino acids, and form a seven-α-helix bundle comprising the central core of the receptor. This global structure is common to all GPCRs (Figure 2B). The TMs are linked by six loops, three situated outside the cell and three in the cytoplasm (Figure 2A and B). This structure has been confirmed by the recent and

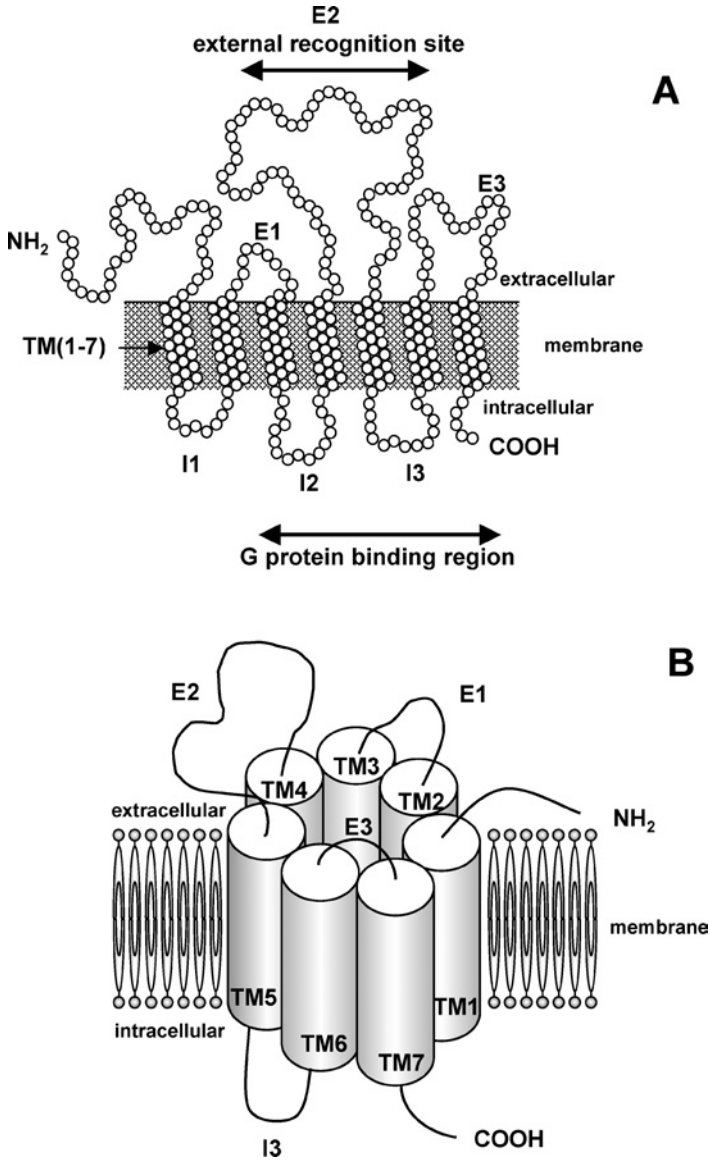

FIG. 2. Generalized olfactory receptor (Or) structure, a specific example of a G-protein coupled receptor. A: amino acid representation showing the seven transmembrane domains (TM), the three extracellular loops (E), and the three intracellular loops (I). Regions that may be involved in ligand binding or G-protein coupling are indicated with a double arrow. B: Or three-dimensional representation in the membrane.

first crystal structure determination of a GPCR, the bovine rhodopsin (Palczewski et al., 2000). To date, this remains the only structure determination of a GPCR.

GPCRs bind a wide number of ligands that range from small organic molecules, nucleotides, lipids, and peptides, to macromolecules such as proteins. Little is known about the exact sites of ligand binding. Candidate areas for ligand binding may include the pocket formed by the membrane spanning segments as well as the external loops (Figure 2B). Length variations in loops and terminal fragments could contribute to functional specificity for ligands and G-proteins (Palczewski et al., 2000). In particular, the second extracellular loop of rhodopsin makes extensive contacts with many extracellular regions as well as with the ligand retinal. Upon binding, the receptor undergoes conformational modifications such as changes in TM orientation and in the structure of the internal loops. These modifications lead to a more open conformation near the receptor G-protein binding site. G-protein activation then induces a cascade of intracellular reactions.

DISCOVERY OF *DROSOPHILA* ODORANT RECEPTOR GENES

Although known vertebrate Ors belong to the GPCR family, they present poor primary sequence identity between vertebrates and the invertebrate *C. elegans*. Perhaps due to these observations, identification of insect Ors has never been achieved by using homology strategies. This suggests that Ors in insects may represent a class of genes unrelated to vertebrates and other invertebrates.

With the beginning of the *D. melanogaster* genome project, which permitted assessment of genomic sequences, several research groups identified a large family of candidate *Drosophila* odorant receptor (*DOr*) genes (Clyne et al., 1999a; Gao and Chess, 1999; Vosshall et al., 1999). Based on the hypothesis that insect Ors should belong to the GPCR family, the three research groups used different algorithms to search for coding exons that encode seven TM proteins. Indeed, as TM domains mainly consist of hydrophobic amino acids, deduced proteins were selected based on their hydropathy profile. Since *Ors* should be expressed in the *Drosophila* olfactory organs (e.g., the antennae and maxillary palpi), candidate genes obtained were subjected to tissue-specific expression analyses. In particular, RNA *in situ* hybridization (ISH) allows specific transcript detection by using a labeled antisense RNA probe that hybridizes to the endogenous mRNA. The three independent approaches brought overlapping data resulting in the discovery of 19 candidate *Drosophila Or* genes, named *Or1–Or19*. These results were later extended with the completion of the *Drosophila* genome (Adams et al., 2000), resulting in the identification of 61 *DOr* genes, each containing seven TM GPCRs (Vosshall et al., 2000; Stocker, 2001). A recent analysis of the updated *Drosophila* genomic sequences (Release 3.1) predicted 62 Or proteins encoded by 60 *Or* genes (Robertson et al., 2003). A standardized nomenclature based on the chromosomal

location of the genes has been established for the *DOr* genes by the *Drosophila* Odorant Receptor Nomenclature Committee (2000).

These *Or* genes encode rather hydrophobic proteins of 370–400 amino acids. Or83b (see "The Particular Case of *Or83b* and its Orthologs"), which possesses 486 amino acids, is an exception. There is a low degree of sequence similarity among the *DOr* genes (17–26% identity), but there are some subfamilies with higher sequence similarity (40–60%). The strongest degree of sequence conservation among genes in the *DOr* family is observed on the 3′ regions that code for TM numbers 6 and 7. No sequence similarity was found between *DOr* genes and the vertebrate or *C. elegans Or* genes (Troemel et al., 1995; Mombaerts, 1999), which explains the failure of the homology-based cloning strategy. In fact, the invertebrate *Ors* form an independent gene family from the vertebrate family. However, their GPCR structure shows similarities with vertebrate Ors, suggesting their functional significance. In particular, DOrs exhibit a relatively large second extracellular loop, as revealed by statistical analyses of length of the receptors (Otaki and Yamamoto, 2003) (Figure 2A). This loop could assist in ligand binding, as suggested from the rhodopsin crystal structure (Palczewski et al., 2000), or alternatively could play a role in dimerization of receptors (Vosshall et al., 2000) (see "The Particular Case of *Or83b* and its Orthologs").

In parallel, the combined efforts of Clyne et al. (2000), Scott et al. (2001), Dunipace et al. (2001), and Robertson et al. (2003) led to the identification of a total of 68 gustatory receptor (Gr) proteins encoded by 60 genes (for a review on *Drosophila* gustatory receptors, see Chyb, 2004). In addition to phylogenetic interrelationship between Ors and Grs (see "Genetic Data, Molecular Evolution, and Developmental Aspects"), four *Gr* genes were found to be expressed in subsets of neurons in the antennae and/or maxillary palpi (Dunipace et al., 2001; Scott et al., 2001). Moreover, neurons expressing one of these *Grs* project axons to glomeruli in the antennal lobe (Scott et al., 2001), suggesting that these Grs may in fact function as Ors.

OR IDENTIFICATIONS IN OTHER INSECT SPECIES

With the discovery of *Drosophila* Or candidates that belong to the GPCR family, it could be postulated that the fundamental molecular nature of Ors, and in particular their GPCR structure, should be conserved across phyla. In addition to pharmacological and immunological evidence for GPCR activity upon odor stimulation in insect antennae (see "Exploratory Investigations of Olfactory Receptors in Insects"), a cDNA coding for a Gq α subunit has been cloned from the antennae of the noctuid moth, *Mamestra brassicae*, and its expression is associated with olfactory sensilla (Jacquin-Joly et al., 2002), suggesting the occurrence of functional GPCRs in moth antennae.

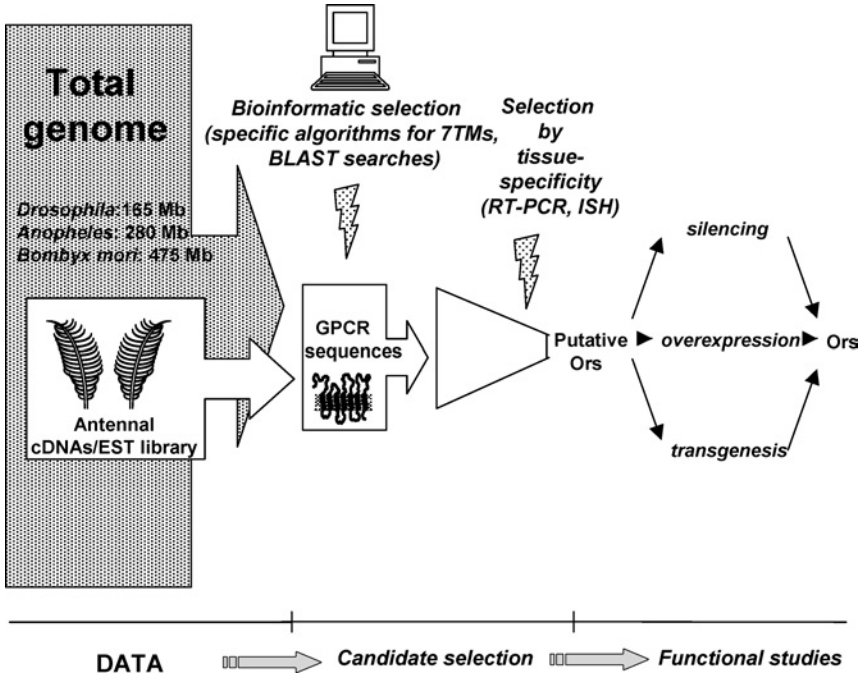

FIG. 3. General procedure used to identify potential *Ors* from insect genomic or cDNA sequence information. Bioinformatics is used to select potential expressed genes that code for proteins with seven TMs. Among these candidates, only genes expressed in olfactory organs are further considered. Functional studies are the final step, and serve to prove involvement in the olfactory process and allow ligand(s) determination. (EST: Expressed sequence tag; ISH: *in situ* hybridization; RT–PCR: Reverse transcription–polymerase chain reaction).

The same protocol as the one used for *Drosophila* (Figure 3), can then theoretically be applied to any insect species to isolate and characterize Ors.

1). *In silico* cloning of candidate GPCR sequences through bioinformatic analyses of sequence databases.
2). Selection among GPCR sequences via expression studies, e.g., reverse transcription–polymerase chain reaction (RT–PCR) on mRNA preparations of different tissues, *in situ* hybridization with olfactory tissues, etc.
3). Final determination through functional studies.

This general scheme, however, prerequires the availability of sequence data that could be achieved through international consortia for whole genome sequencing. Sequence data could also come from smaller-scale efforts such as EST

(expressed sequence tag) libraries developed from expressed genes in a particular tissue, or proteome analysis.

Up to now, the use of genomic data has allowed identification of Or candidates in several other insect species, such as the malaria vector mosquito, *Anopheles gambiae* (Fox et al., 2001, Hill et al., 2002; Pitts et al., 2004), the tobacco budworm, *Heliothis virescens* (Krieger et al., 2002, 2004), and the honey bee, *Apis mellifera* (Robertson, personal communication).

Taking advantage of the initiation of the *A. gambiae* genome sequencing by Genoscope (France), Fox et al. (2001) identified the first candidate odorant receptors from a nondrosophilan insect. Postulating that a similar family of seven TM receptors would also mediate odorant signaling in this species, they used a bioinformatic-homology-based approach to analyze the database for sequences similar to *Drosophila Ors*. Five genes were identified that were selectively expressed in olfactory organs (Fox et al., 2001, 2002; Pitts et al., 2004). After the *A. gambiae* genome sequencing project was completed, a total of 276 GPCRs were identified by using bioinformatics, mainly based on amino acid physico-chemical information (Hill et al., 2002). Among them, 79 candidate odorant receptors (named GPRors or AgOrs) were characterized for tissue expression and, along with 76 candidate gustatory receptors, for their molecular evolution relative to *Drosophila* (Hill et al., 2002). Eighty percent of the *AgOrs* show expression only in olfactory tissues, whereas four *AgOrs* show additional expression in legs. Scott et al. (2001) had previously reported that some *Drosophila Ors* are expressed in both antennae and legs. At least five of the characterized *AgOrs* display female-specific expression. As observed for *DOrs*, sequence similarity is low among the *AgOrs*.

A similar approach was used by Krieger et al. (2002, 2004) in the noctuid moth, *H. virescens*. Access to the nonpublic *H. virescens* genomic database from Genoptera (USA) allowed Krieger et al. (2002, 2004) to characterize a divergent gene family coding for the first candidate Ors in a lepidopteran crop pest. In total, 21 candidates were described in this species. Each receptor subtype appears to be expressed in a distinct population of sensory cells, as already observed for *Drosophila* as well as vertebrate *Ors* (see "Odor Coding: From Stereochemical Information to an Olfactory Sensory Map in the Brain"). A small group of three receptors share >40% identity and are expressed exclusively in male moths in pheromone-sensitive neurons, making them good candidate pheromone receptors.

Through a preliminary evaluation of the first draft of the *A. mellifera* genome, H. M. Robertson (personal communication) identified about 60 potential *Ors* in this species.

Ors from other insect species have also been identified through systematic sequencing of antennal expressed genes in EST library projects. Using this approach, Whitfield et al. (2002) discovered one potential *Or* from *A. mellifera* that appeared as an *Or83b* homolog (see "The Particular Case of *Or83b* and its

Orthologs"), Newcomb et al. (2003) identified sequences from the tortricid moth, *Epiphyas postvittana*, and Patch and Robertson (personal communication) discovered two others in the hawkmoth, *Manduca sexta*. However, since potential *Ors* are expressed at very low levels in the antennae, such a strategy often requires sequencing of several thousand ESTs with only several potential *Or* sequences being obtained. Another approach is to use normalized or subtractive libraries, in which rare transcripts are enriched.

To date, a complete list of *Or* genes has only been achieved in *Drosophila* and *Anopheles* through genomic data analyses. Even with these *Or* sequences in hand, the divergence of these receptors has not allowed *Or* identification in other species using homology-based PCR. Indeed, comparison of the *Or* sequences shows how divergent they are, even among species in the same insect order. From the literature, international databases, and personal communication, a nonexhaustive list of candidate Ors encompasses 4 insect orders, 10 families, and 12 species (Table 1).

THE PARTICULAR CASE OF *Or83b* AND ITS ORTHOLOGS

Although most homology-based strategies have failed to identify *Ors* in other species, Krieger et al. (2003) amplified one candidate *Or* subtype in several insect species from various orders. These included the honey bee, *A. mellifera* (Hymenoptera), the blowfly *Calliphora erythrocephala* (Diptera), and the yellow mealworm, *Tenebrio molitor* (Coleoptera). The candidate sequences showed high amino acid sequence conservation with *Drosophila* Or83b and AgOr7. Such PCR experiments have also allowed identification of homolog cDNAs in other species. For example, we identified a homolog *Or* cDNA in the noctuid *M. brassicae* (unpublished results), and Melo et al. (2004) cloned the *AaOr7* from the yellow fever mosquito, *Aedes aegypti*. These Ors share around 60–80% identity and could be considered as orthologs, defining an unusual receptor subtype highly conserved across insect orders (Figure 4). Dunipace et al. (2001) noted that among Ors, Or83b appears most similar to the Gr family proteins, and this conclusion is supported by phylogenetic analysis (Robertson et al., 2003). In addition to this unusually high degree of conservation across insect orders, the *Or83b* orthologs are all expressed in a large number of cells of the antennae, palpi, and proboscis (Clyne et al., 1999a; Vosshall et al., 2000; Krieger et al., 2003; Melo et al., 2004; Pitts et al., 2004). In *Drosophila*, *Or83b* is expressed in addition to the single specific *Or* typically expressed in each neuron. Therefore, this unique receptor subtype has been proposed to fulfill a special function common to all chemosensory neurons of insects. Although it may not function as a typical Or recognizing particular ligands, it could be involved in Or activation, for example, as a dimerization partner (Vosshall et al., 2000; Krieger et al., 2003). Heterodimerization is now well documented for nonolfactory GPCRs, such as GPCRs for transmitters and peptides where heterodimerization could modulate the binding specificity of

TABLE 1. INSECT OLFACTORY RECEPTOR CANDIDATES

Order Family	Species	Names	Database accession no.	References	Expression[a]	Data sources
Diptera	*Drosophila*	Or2a	NM_080307	Vosshall et al., 2000	A	Genome
Drosophilidae	*melanogaster*	Or7a	NM_078526	Vosshall et al., 2000	A	Genome
		Or9a	NM_078552	Vosshall et al., 2000	A	Genome
		Or10a	NM_078567	Vosshall et al., 2000	A	Genome
		Or13a	NM_078635	Vosshall et al., 2000	A	Genome
		Or19a	NM_080274	Vosshall et al., 2000	A	Genome
		Or22a	NM_078729	Vosshall et al., 2000	A	Genome
		Or22b	NM_058077	Vosshall et al., 2000	A	Genome
		Or23a	NM_078734	Vosshall et al., 2000	A	Genome
		Or33a	NM_078829	Vosshall et al., 2000	A	Genome
		Or33b	NM_078830	Vosshall et al., 2000	A	Genome
		Or35a	NM_165117	Vosshall et al., 2000	A	Genome
		Or42b	NM_078900	Vosshall et al., 2000	A	Genome
		Or43a	NM_078923	Vosshall et al., 2000	A	Genome
		Or43b	NM_078932	Vosshall et al., 2000	A	Genome
		Or47a	NM_078965	Vosshall et al., 2000	A	Genome
		Or47b	NM_078966	Vosshall et al., 2000	A	Genome
		Or49b	NM_078997	Vosshall et al., 2000	A	Genome
		Or56a	NM_079072	Vosshall et al., 2000	A	Genome
		Or59b	NM_079098	Vosshall et al., 2000	A	Genome
		Or65a	NA	Vosshall et al., 2000	A	Genome
		Or65b	NA	Vosshall et al., 2000	A	Genome
		Or65c	NA	Vosshall et al., 2000	A	Genome
		Or67a	NM_079281	Vosshall et al., 2000	A	Genome
		Or67c	NM_079294	Vosshall et al., 2000	A	Genome
		Or69a (=Or69aB)	NA	Vosshall et al., 2000 (modified names by Robertson et al., 2003)	A	Genome
		Or69b (=Or69aA)	NM_079326	Vosshall et al., 2000 (modified names by Robertson et al., 2003)	A	Genome
		Or82a	NA	Vosshall et al., 2000	A	Genome
		Or83c	NM_079520	Vosshall et al., 2000	A	Genome
		Or85a	NM_079553	Vosshall et al., 2000	A	Genome
		Or85b	NM_079555	Vosshall et al., 2000	A	Genome
		Or85f	NM_079565	Vosshall et al., 2000	A	Genome
		Or88a	NM_079624	Vosshall et al., 2000	A	Genome
		Or98a	NM_079812	Vosshall et al., 2000	A	Genome
		Or1a	NM_080290	Vosshall et al., 2000	MP	Genome
		Or33c	NM_078831	Vosshall et al., 2000	MP	Genome
		Or46a (=Or46aA)	NM_078953	Vosshall et al., 2000 (modified names by Robertson et al., 2003)	MP	Genome
		Or59c	NM_079099	Vosshall et al., 2000	MP	Genome
		Or71a	NM_168604	Vosshall et al., 2000	MP	Genome
		Or85d	NM_079557	Vosshall et al., 2000	MP	Genome
		Or85e	NM_079559	Vosshall et al., 2000	MP	Genome
		Or83b	NM_079511	Vosshall et al., 2000	O	Genome
		Or22c	NM_078730	Vosshall et al., 2000	Not expressed	Genome
		Or24a	NM_078746	Vosshall et al., 2000	Not expressed	Genome
		Or30a	NM_078796	Vosshall et al., 2000	Not expressed	Genome
		Or42a	NM_078898	Vosshall et al., 2000	Not expressed	Genome
		Or45a	NM_176115	Vosshall et al., 2000	Not expressed	Genome
		Or45b	NM_078943	Vosshall et al., 2000	Not expressed	Genome
		Or46b (=Or46aB)	NM_165752	Vosshall et al., 2000 (modified names by Robertson et al., 2003)	Not expressed	Genome
		Or49a	NM_078987	Vosshall et al., 2000	Not expressed	Genome
		Or59a	NM_079097	Vosshall et al., 2000	Not expressed	Genome
		Or63a	NM_079171	Vosshall et al., 2000	Not expressed	Genome
		Or74a	NW_047330	Vosshall et al., 2000	Not expressed	Genome
		Or88a	NM_079624	Vosshall et al., 2000	Not expressed	Genome
		Or85c	NM_079556	Vosshall et al., 2000	Not expressed	Genome
		Or92a	NM_079690	Vosshall et al., 2000	Not expressed	Genome
		Or94a	NM_079731	Vosshall et al., 2000	Not expressed	Genome
		Or94b	NM_079732	Vosshall et al., 2000	Not expressed	Genome
		Or98b	NM_079816	Vosshall et al., 2000	Not expressed	Genome
		Or67b	NM_079283	Vosshall et al., 2000	U	Genome
		Or67d	NM_140133	Vosshall et al., 2000	U	Genome
		Or19b	NM_167690	Robertson et al., 2003		

TABLE 1. CONTINUED.

Order Family	Species	Names	Database accession no.	References	Expression[a]	Data sources
Diptera Culicidae	*Anopheles gambiae*	AgOr1	AF364130	Fox et al., 2001	O	Genome
		AgOr2	AF364131	Fox et al., 2001	O	Genome
		AgOr3 & 4	AF364132	Fox et al., 2001	O	Genome
		AgOr5	AY062432	Fox et al., 2002	O	Genome
		79 GPRors		Hill et al., 2002		Genome
		AgOr7	AY363725	Pitts et al., 2004	A, MP, p	Genome
	Aedes aegypti	AaOr7	AY582943	Melo et al., 2004	A,MP,L,p	Homology cloning
Diptera Calliphoridae	*Calliphora erythrocephala*	CeryR2	AJ555538	Krieger et al., 2003	A	Homology cloning
Lepidoptera Noctuidae	*Heliothis virescens*	HR1	AJ487476	Krieger et al., 2002	A,P	Genome
		HR2	AJ487477	Krieger et al., 2002	A,P	Genome
		HR3	AJ487478	Krieger et al., 2002	A,P	Genome
		HR4	AJ487479	Krieger et al., 2002	A,P,w,ab	Genome
		HR5	AJ487480	Krieger et al., 2002	A,P,l	Genome
		HR6	AJ487481	Krieger et al., 2002	A,P,l,w,t ab	Genome
		HR7	AJ487482	Krieger et al., 2002	A	Genome
		HR9	AJ487484	Krieger et al., 2002	A	Genome
		HR8	AJ487483	Krieger et al., 2002	A,P, T	Genome
		HR10	AJ748325	Krieger et al., 2004		Genome
		HR11	AJ748326	Krieger et al., 2004	Am,Af	Genome
		HR12	AJ748327	Krieger et al., 2004		Genome
		HR13	AJ748328	Krieger et al., 2004	Am,p,l,w,ab	Genome
		HR14	AJ748329	Krieger et al., 2004	Am	Genome
		HR15	AJ748330	Krieger et al., 2004	Am	Genome
		HR16	AJ748331	Krieger et al., 2004	Am	Genome
		HR17	AJ748332	Krieger et al., 2004		Genome
		HR18	AJ748333	Krieger et al., 2004		Genome
		HR19	AJ748334	Krieger et al., 2004		Genome
		HR20	AJ748335	Krieger et al., 2004		Genome
		HR21	AJ748336	Krieger et al., 2004		Genome
Lepidoptera Noctuidae	*Mamestra brassicae*	MbraR2	AY485222	Jacquin-Joly et al., 2003, direct submission	A, p	Homology cloning
Lepidoptera Bombycidae	*Bombyx mori*	BmorR2	AJ555487	Krieger et al., 2003	A	Homology cloning
Lepidoptera Saturniidae	*Antheraea pernyi*	AperR2	AJ555486	Krieger et al., 2003		Homology cloning
Lepidoptera Sphingidae	*Manduca sexta*	2 Ors	NA	Patch and Robertson, personal communication		EST[b]
Lepidoptera Tortricidae	*Epiphyas postvittana*	3 Ors	NA	Newcomb et al., 2003		EST
Hymenoptera Apidae	*Apis mellifera*	AmelR2	AJ555537	Krieger et al., 2003		Homology cloning
		= *Or83b* ortholog ~60 Ors		Whitfield et al., 2002 Robertson, personal communication		EST genome
Coleoptera Tenebrionidae	*Tenebrio molitor*	TmolR2	AJ555539	Krieger et al., 2003		Homology cloning

[a] Abbreviations—O: olfactory tissues (A: antennae and M.P.: maxillary palpi); P: proboscis; L: legs; W: wings; T: thorax; Ab: abdomens; m: male; f: female; U: unknown expression pattern. Capital letters: high expression, small letters: low expression. NA: not available.

[b] EST: expressed sequence tag project.

the conventional receptor (Jordan and Devi, 1999; Pfeiffer et al., 2001), or as described for the GABA BN1/B2 receptor dimer (Robbins et al., 2001) (for a review on GPCR dimerization, see Terrillon and Bouvier, 2004). However, the *Drosophila* Or43a appeared to respond to odorants when expressed in *Xenopus laevis* oocytes where there is no Or83b expression (Wetzel et al., 2001), leaving

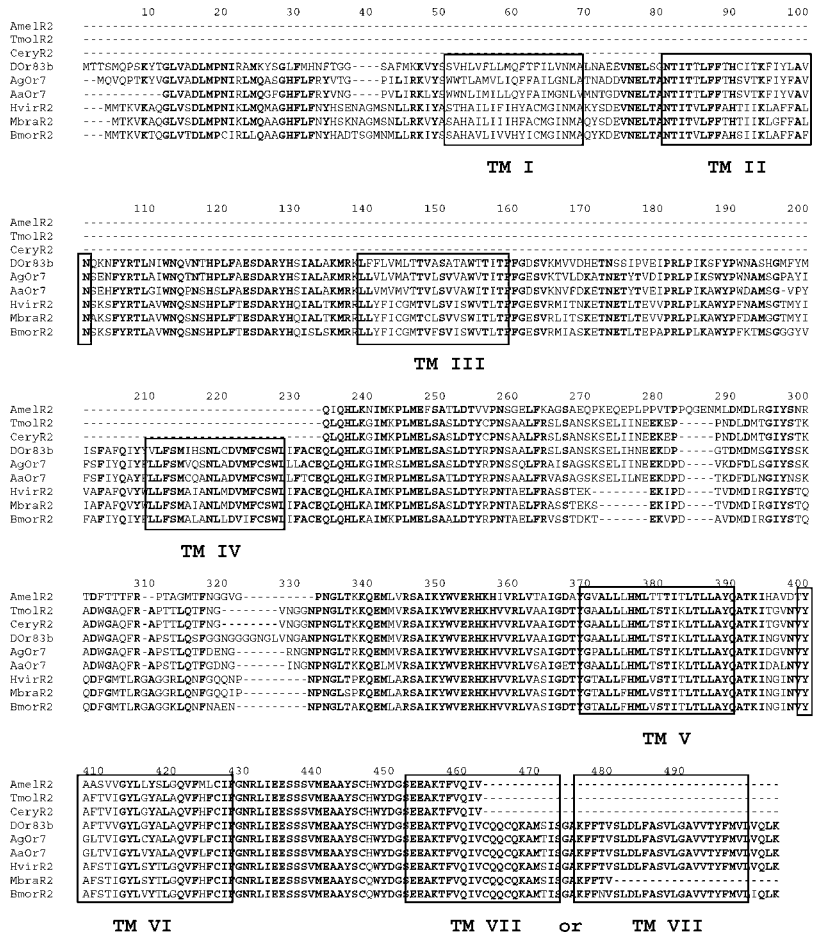

FIG. 4. Amino acid alignment of the DOr83b ortholog receptors from nine insect species: a unique example of a conserved insect Or family. Conserved residues shared by all or all but one species are bold. The seven transmembrane domains (TM) are boxed. Two possible TM VII positions have been proposed (Fox et al., 2001; Pitts et al., 2004). Accession numbers and names of the sequences used in this figure are listed in Table 1.

the question of Or83b heterodimerization partner unresolved (see "Or Functional Studies").

DISCOVERY OF OTHER NEURONAL RECEPTOR TYPES: SNMPs AND GCs

The Sensory Neuron Membrane Proteins (SNMPs). Before the discovery of the *DOr* genes, Rogers et al. (1997) purified and cloned an abundant protein in

A. polyphemus ORN membrane extracts, during a search for membrane proteins that might support olfactory mechanisms in Lepidoptera. Molecular and immunological analyses suggest that this protein, named SNMP-1 for sensory neuron membrane protein, is uniquely expressed in cilia, dendrite, and cytosolic granules of the soma of ORNs (Rogers et al., 1997, 2001a). Sequence analysis has revealed the presence of two TMs with a large extracellular loop, and homologies with vertebrate proteins from the CD36 family, a phylogenetically diverse family of receptor-like membrane proteins implicated in diverse functions, such as endocytosis or binding to apoptotic cells (e.g., Ohgami et al., 2001). SNMP-1 homolog proteins were then found in diverse lepidopteran species, including *M. sexta* (Rogers et al., 2001b). In the latter species, these workers also identified a second SNMP type (SNMP-2) that shared the same characteristics as SNMP-1 (two TMs and localization restricted to ORNs), although SNMP-2 was quite divergent in sequence, with only 27% identity with SNMP-1. These results highlighted SNMP diversity within species.

Although the function of SNMP in the olfactory process is not yet understood, several hypotheses have been proposed (Rogers et al., 2001a) (Figure 5). A first hypothesis is that SNMPs could act as Ors in Lepidoptera, since a previous study that used a photoaffinity analog of the pheromone identified a pheromone-binding membrane protein of similar size and tissue distribution (Vogt et al., 1988). SNMP could bind either the pheromone directly or the pheromone–PBP complex. However, since all the Ors known to date belong to the GPCR family, this hypothesis was not further extended. The observed interaction with pheromone analogs could have resulted from an alternative or complementary process to GPCR binding.

Alternatively, due to the SNMP homologies with CD36 and the presence of a big extracellular loop, they may interact with proteinaceous ligands (like OBPs) and act as docking proteins for the odorant–OBP complex. This may facilitate access of the odorant to the Or (Figure 5A).

A third possibility is that SNMPs interact with other dendritic proteins, such as the Ors, to form active heterodimer complexes (Figure 5B). Finally, SNMPs may act as scavengers, allowing internalization of odorants, OBPs or odorant–OBP complexes. This would lead to metabolism of OBPs or the complexes by intracellular enzymes (Figure 5C) (Vogt, 2003). Other possible functions in the neuron signaling process may occur and have yet to be tested by functional studies.

The Guanylyl Cyclases (GCs). This family of one TM protein hydrolyzes guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). GCs have been identified in vertebrate as well as invertebrate species, including *C. elegans* and insects. Some have been proposed to be implicated in odorant–pheromone reception or its regulation. Indeed, *C. elegans* GCs are specifically expressed in the chemosensory neurons (Yu et al., 1997), and overexpression of one of them, ODR-1, modifies odorant discrimination and olfactory adaptation

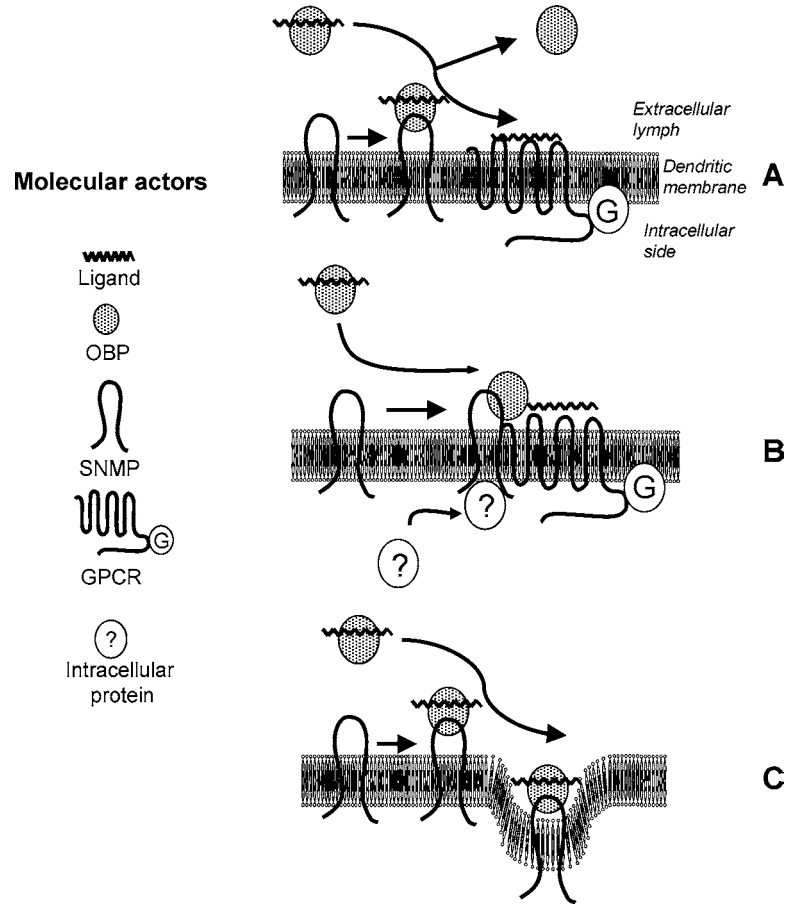

FIG. 5. Proposed functions for SNMP (from Rogers et al., 2001a,b). A: SNMP may facilitate access of the odor–OBP complex to a classical GPCR. B: SNMP may complex with Or and/or other intracellular proteins, contributing to odor or odor–OBP complex reception. C: SNMP may have scavenger properties, related to odor or odor–OBP complex internalization.

(L'Etoile and Bargmann, 2000). In the moth, *M. sexta*, cGMP immunoreactivity and soluble GC have been localized in the antennal sensilla (Stengl et al., 2001), suggesting that the intracellular messenger cGMP plays a role in olfactory transduction. In addition, Tanoue et al. (2001) reported the isolation of a GC receptor type cDNA from male *B. mori* antennae. Immunohistochemical study revealed that the protein localizes in the antennal lobe glomerulus and in the soma and axon of sensory neurons (Tanoue et al., 2001). A novel type of GC, cloned in *M.*

sexta, appears to be expressed in the cell bodies and dendrites, but not axons, of ORNs (Nighorn et al., 2001). So far, odorant binding on the extracellular portion of GC receptors has not been observed, and their role in the olfactory process is not yet understood.

GENETIC DATA, MOLECULAR EVOLUTION, AND DEVELOPMENTAL ASPECTS

Gene Structures and Genome Localization. Contrary to monoexonic mammalian *Ors*, insect and *C. elegans Or* genes (Troemel et al., 1995) carry introns. Some *Drosophila Or* genes may undergo alternative splicing (Robertson et al., 2003), as noted for several *Gr* genes in *Drosophila* (Clyne et al., 2000) and *A. gambiae* (Hill et al., 2002).

In *Drosophila*, the majority of *Or* genes are spread widely through the genome, indicating that they are old members of a gene family that have been distributed around the genome by the processes of genome flux (Robertson et al., 2003). This ancient origin of the chemoreceptor family is also supported by intron evolution analysis, as well as the observation of an extreme divergence within the family (Robertson et al., 2003). Some genes are still clustered, indicating relatively recent gene duplication. The *Drosophila Or* genomic distribution is in contrast with the pattern observed with the mammalian *Ors* (Zhang and Firestein, 2002) and the *C. elegans* chemoreceptor genes (Robertson, 2001), which are highly clustered on particular chromosomes, reflecting the relatively recent expansions of these chemoreceptor families.

Comparing the molecular evolution of *Drosophila* and *A. gambiae Or* genes has revealed the expansion of gene subfamilies unique to each dipteran lineage. In *A. gambiae*, the *Or* family members are dispersed among the three chromosomes, with most *AgOrs* tightly linked as pairs, triplets, or larger clusters of up to nine genes, whereas 17 *AgOrs* exist as single genes (Hill et al., 2002). The large clusters of *AgOr* genes are exclusively made up of recently duplicated genes. This pattern of lineage-specific gene subfamily expansion may reflect the ecological and physiological relevance of these receptors: they may be responsible for detection of signals uniquely important to each species, like fruit odors for *D. melanogaster* and human host odors for *A. gambiae* (Hill et al., 2002). The high divergence between *Or* sequences from insects, nematodes, and vertebrates suggests that they have an independent origin, arising from an independent mechanism of evolution (Vosshall et al., 1999).

Scott et al. (2001) introduced the notion that the *Drosophila Or* and *Gr* families are evolutionarily related in a chemoreceptor superfamily, and this is supported by phylogenetic analysis (Robertson et al., 2003). In addition, the presence of antennal Grs that may function as Ors (see "Discovery of *Drosophila* Odorant Receptor Genes") suggests that *Or* function has evolved separately several times within the superfamily.

Regulation of Or Gene Expression. A common feature between vertebrate and invertebrate *Ors* is the hypothesis that one neuron expresses only one *Or* type (see "Odor Coding: From Stereochemical Information to an Olfactory Sensory Map in the Brain"). Although this hypothesis has been confirmed in mammals and in *Drosophila* (Malnic et al., 1999; Vosshall et al., 2000), *C. elegans* appears to be an exception, since more than one type of *Ors* is expressed in a given sensory neuron (Troemel et al., 1995).

Little is known about the regulatory processes that underly expression of only one *Or* type per neuron. The mechanism of segregation could involve regulatory elements situated near the transcription initiation sites of *Or* genes, which would receive specific information about the *Or* expression area (Qasba and Reed, 1998).

In *Drosophila*, expression of abnormal chemosensory jump 6 (Acj6), a transcription factor, determines the receptive odorant profiles of ORNs, and then may contribute to the choice of the receptor gene to be expressed (Clyne et al., 1999b). An unusual mode of gene expression, that involves a mutually exclusive expression of odorant receptors, has been recently demonstrated in mice (Serizawa et al., 2000).

Developmental Aspects and Sexual Dimorphism. Few studies have investigated the temporal expression pattern of *Or* genes from embryonic development to adult stage. In *Drosophila*, Vosshall et al. (1999) reported an absence of *DOr* gene expression at any stage during embryonic development. To our knowledge, *Drosophila* larval stages were not studied. Some studies have investigated *DOr* expression during pupal development, hypothesizing that *Or* genes might have a role in guiding the axons of the olfactory neurons to the correct glomeruli. Indeed (see "Other Possible Functions for Ors"), vertebrate *Or* genes have been proposed to play such a role in development (Mombaerts et al., 1996; Wang et al., 1998). Although different members of the *DOrs* family initiate expression at different times during antennal development (Clyne et al., 1999a), *DOrs* seem to be expressed only after the establishment of synaptic connections (Vosshall et al., 2000).

One *A. gambiae* putative *Or* (*AgOr7*) has been extensively studied from a developmental point of view (Pitts et al., 2004). Although the most robust expression was observed in adult olfactory organs, this gene appeared to be expressed during preimago stages, including early stage larvae, late stage larvae, and pupae, but not in the embryos. In *A. polyphemus* and *M. sexta*, another class of membrane proteins (the SNMPs) are expressed late in adult development and into adult life (Rogers et al., 1997, 2001b). At this point, morphogenesis has been completed and olfactory neurons are functional.

Differential expression of *Or* in female or male olfactory organs may be correlated with functional differences between the sexes. Although *DOrs* do not appear to be sexually dimorphic in *Drosophila* organs, at least five female-specific *Ors* have been identified in *A. gambiae* (Fox et al., 2001; Zwiebel and Takken, 2004). In mosquitoes, host selection and blood feeding are restricted

to females. Thus, female-specific expression of *Ors* may be indicative of a role in establishing host preference (Fox et al., 2001). In *Drosophila*, a putative gustatory receptor, Gr68a, is expressed in chemosensory neurons of male-specific gustatory bristles in the forelegs, with no expression in females (Bray and Amrein, 2003). In addition, Gr68a expression is dependant on the sex determination gene, *doublesex*. These considerations are consistent with a function in pheromone recognition in *Drosophila* since males perceive the female nonvolatile pheromone during courtship (Coyne et al., 1994). Indeed, molecular analyses of *Gr68a* proved that it is required for normal male courtship (Bray and Amrein, 2003) (see "Future Perspectives, Opportunities, and Challenges of Insect or Studies").

Very recently, a male-exclusive expression of three putative Ors from the moth *H. virescens* lead to the identification of the first good lepidopteran pheromone receptor candidates (Krieger et al., 2004).

SIGNAL TRANSDUCTION IN INSECT SENSORY CELLS

Olfactory transduction has been reviewed in great depth by Krieger and Breer (1999). Here, we will only give a brief overview of how Or activation could lead to the generation of an electrical signal. The different elements involved in this transduction cascade are also involved in other diverse phenomena, such as adaptation of the ORNs (as reviewed for vertebrates by Zufall and Leinders-Zufall, 2000).

The binding of the odorant to its specific receptor situated in the dendritic membrane leads to the activation of a G-protein, which in turn mediates the response via intraneuronal second messengers that trigger the opening of ion channels and local depolarization. Ultimately, this elicits an action potential in the neuron (Figure 6). G-proteins are heterotrimers, comprising α, β, and γ subunits. The α subunit is responsible for GTP binding and hydrolysis to GDP. G-proteins are generally referred to by this α subunit, since it is hypothesized that it confers the G-protein specificity: for example Gq proteins activate phospholipase C (PLC), whereas Gs activate adenylyl cyclase. From biochemical, electrophysiological, and molecular data, the current hypothesis is that the PLC–InsP3 reaction cascade may be the major pathway for signal transduction in insect olfactory neurons. Indeed, Gqα3 mediates odor responses in *Drosophila*, as recently demonstrated by Kalidas and Smith (2002), who targeted Gqα3 silencing in ORNs by RNA-mediated interference (RNAi) (see "Future Perspectives, Opportunities, and Challenges of Insect or Studies"). Using the *Or83b* promoter, they expressed the silencing construct in a large fraction of ORNs leading to olfactory defects *in vivo* at both the physiological and the behavioral levels.

PLC mediates the hydrolysis of inositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-triphosphate ($InsP_3$) and diacyl glycerol (DAG) (Krieger and Breer, 1999).

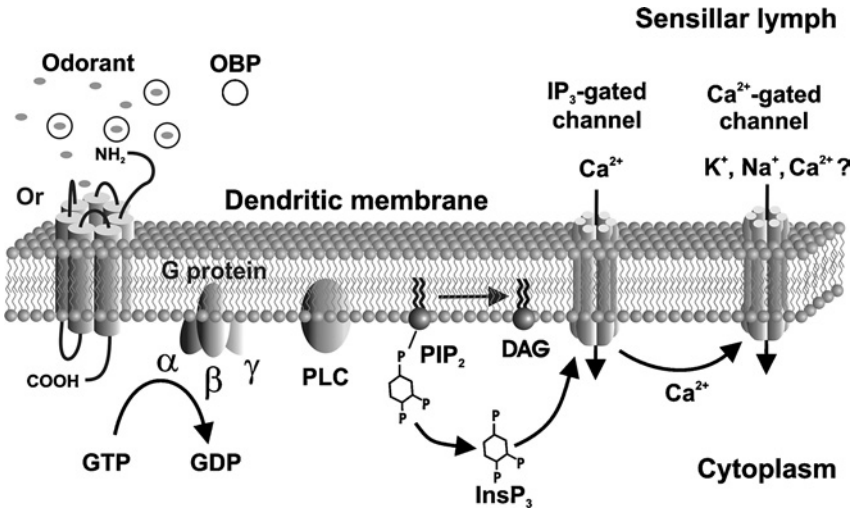

FIG. 6. Hypothetical olfactory transduction cascade in insect olfactory neurons. Ligand binding to the olfactory receptor (Or) activates a G-protein, which in turn activates a phospholipase C (PLC). PLC mediates the hydrolysis of PIP_2 (inositol 4,5-biphosphate) into $InsP_3$ (inositol 1,4,5-triphosphate) and DAG (diacyl glycerol). Downstream events then lead to ion channel opening and membrane depolarization.

The downstream events that lead to membrane depolarization are not yet well understood. $InsP_3$ could act on IP_3-gated ion channels leading to Ca^{2+} entry in the cell, which in turn act on Ca^{2+}-gated cationic ion channels (Figure 6). These inward currents form a receptor potential that elicits a discharge of action potentials that travel down ORN axons to the first relay in the brain, the AL, encoding odor quantity and quality.

ODOR CODING: FROM STEREOCHEMICAL INFORMATION TO AN OLFACTORY SENSORY MAP IN THE BRAIN

Olfactory messages are generally composed of blends of molecules. Odorants are perceived as a precise mixture, which is particularly exemplified by pheromonal blends used by insects for intraspecific communication (see the Pherolist: Witzgall et al., 2004).

With approximately 1,300 antennal ORNs, 120 maxillary palp ORNs, and 62 Ors, *Drosophila* is able to recognize and discriminate among a large number of distinct odorants. How is the combination of the stereochemical information detected by ORNs transferred to the higher centers of the nervous system? In mammals, individual ORNs express only 1 of 1,000 receptor genes, and the axons from ORNs expressing a specific receptor converge upon two

spatially invariant glomeruli within the olfactory bulb (reviewed and discussed in Mombaerts, 2004). The quality of the stimulus is, therefore, encoded by specific combinations of activated glomeruli. An olfactory sensory map in the mammalian olfactory bulb corresponds to a spatial map of Or expression in the sensory epithelium.

Although such a logic of olfactory discrimination is not observed in the invertebrate *C. elegans*, where each chemosensory cell expresses a large number of *Or* genes (Troemel et al., 1995), this functional correspondence appears to be conserved between insects and mammals, as illustrated below for *Drosophila*.

Maps of Drosophila Olfactory Receptors in the Antennae. DOr gene expression patterns were analyzed by RT–PCR and RNA ISH in the two olfactory sensory organs of the adult fly (e.g., the third antennal segment and the maxillary palpus) (Gao and Chess, 1999; Clyne et al., 1999a; Vosshall et al., 1999, 2000). Expression of *DOr* genes is specifically localized either in the antenna (39 *DOr* genes), or in the maxillary palpus (9 *DOr* genes), or in both organs (in the case of *Or83b*), with little variation according to the techniques used by different research groups (Gao and Chess, 1999; Clyne et al., 1999a; Vosshall et al., 1999, 2000) (Table 1). Some *Ors*, however, were not detected, perhaps due to their low level of expression or, alternatively, they may be expressed in other tissues or during different times during development. Within the olfactory organs, *DOr* genes are selectively expressed in small subsets of ORNs, which appear bilaterally symmetric and spatially conserved between individuals (Vosshall et al., 1999, 2000). Furthermore, Vosshall et al. (1999, 2000) demonstrated that there are nonoverlapping expression patterns between the *DOr* genes in the antenna or in the maxillary palpus. This suggests that each *DOr* is expressed selectively in a small subset of sensory cells that is spatially defined within the antenna and maxillary palpus. Each ORN expresses one of the *DOr* genes and is, therefore, functionally distinct (Vosshall et al., 1999, 2000). The number of ORNs expressing a given *DOr* type varies from 2 to 50, with an average of 25. The ubiquitous *Or83b* gene appears as an exception since it is expressed in approximately two-thirds of all ORNs and co-expressed in these ORNs with another *Or* type (Vosshall et al., 1999; Kalidas and Smith, 2002) (see "The Particular Case of *Or83b* and its Orthologs").

The spatial map of *Or* expression in olfactory organs approximates the number and distribution of different functional types of neurons in the antennae and maxillary palpi defined by electrophysiology (de Bruyne et al., 1999, 2001), suggesting that the *DOr* genes indeed encode the ligand-binding odorant receptors in *Drosophila* (Vosshall, 2001).

A Sensory Map in the Brain. The anatomy of the insect olfactory system resembles that of vertebrates. Insect ORN axons project to the ALs, the equivalent of the vertebrate olfactory bulb. The ALs are organized in synaptic regions called

glomeruli, which are the first odorant processing areas. Olfactory information is then relayed via antennal lobe projection neurons, the equivalent of the vertebrate mitral cells, to both the mushroom bodies, and the lateral horn of the protocerebrum (Stocker, 1994; Ito et al., 1998). The coding of sensory stimuli into specific patterns of neuronal activity generates an internal representation of the external world that is processed by brain centers to elicit complex sensory responses. Many studies have provided evidence that each individual ORN projects to a single glomerulus within the AL. In *Drosophila*, the availability of *Or* genes has allowed the use of genetic labeling techniques that have revealed that all ORNs expressing a given receptor converge upon one or two AL glomeruli (Gao et al., 2000; Vosshall et al., 2000), whose position and size are invariant among individuals. This conserved topographic map of DOr projections is consistent with previous studies in insects that mapped AL activities after specific olfactory stimulation (Rodrigues, 1988; Galizia et al., 1999). These studies demonstrated that different odorants elicit distinct patterns of glomerular activity in *Drosophila* as well as *Apis mellifera*. The quality of the olfactory stimulus would, therefore, be encoded by the specific combination of glomeruli activated by a given odorant. Activated glomeruli define an odor-specific map that may then be decoded in higher brain centers.

OR FUNCTIONAL STUDIES

Since *DOrs* have been discovered only recently, few studies have reported functional assays of insect Ors. Here, we will first briefly describe the development of functional studies of Ors from vertebrates and the nematode, *C. elegans*, that will allow us to describe the different functional techniques and highlight their difficulties. The major impediment to functional studies is that membrane bound Ors are not water soluble, which makes them difficult to study by using the classical techniques typically used to elucidate structure–function relationships. Then, we will review the first functional studies of Ors in insects.

*O*r Functional Studies Developed in Vertebrates and *C. elegans*

Functional Expression in Heterologous Systems. Vertebrate Ors have been studied extensively by using functional expression in various heterologous systems that do not normally express these proteins. These systems have included mRNA injection in *Xenopus laevis* oocytes, transfection of *Escherichia coli* bacterial cells or HEK eukaryotic cells, and recombinant baculovirus infection of insect cells (e.g., Raming et al., 1993a; Zhang et al., 1997; Krautwurst et al., 1998; Wetzel et al., 1999). However, such experiments have had to overcome obstacles. Since Ors naturally function in the cell plasma membrane, the most difficult obstacle has been to target the heterologous protein to the membrane. Thus, *Or* genes are often fused to a signal sequence that will target the protein to

the cell membrane after expression. In addition, a cDNA encoding a G-protein has to be co-transfected in the cell system to allow coupling with the Or and signal transduction for a chosen pathway. Activation of Or protein upon odorant stimulation then has to be visualized and quantified. Calcium imaging has been used extensively for functional vertebrate Or studies (see the review from Touhara, 2002). Or–ligand interaction leads to intracellular calcium increase (see "Signal Transduction in Insect Sensory Cells"). This calcium increase, an indirect measure of Or activation, is assayed by fluorescence intensity of a probe injected in the cell. Such functional studies, in addition to being difficult, are then performed by using an artificial reconstituted system that may not reflect the natural one. Second, although odorants are volatile airborne molecules, these reconstituted systems require delivery of the odorant in solution, and many functional studies have used odorant concentrations that are higher than physiological concentrations to obtain responding cells (e.g., Wetzel et al., 1999). In addition, the quality of some odorants is perceived differently at different concentrations (Touhara, 2002).

Combination of Calcium Imaging and Single Cell RT–PCR. Touhara et al. (1999) and Touhara (2002) have reported experiments aimed at deciphering odorant–Or pairing by a two-step process. The response profile of an isolated olfactory neuron to a panel of odorants was first examined through calcium imaging. Then, the *Or* expressed in the individual neuron was identified by single cell RT–PCR. In this way, odorant–Or couples were identified in mice (Malnic et al., 1999). Neurons expressing the same Or appear to recognize several odorant molecules with a selectivity that is a function of the size of the chain or the functional group that they are carrying. On the other hand, Malnic et al. (1999) showed that one odorant could be recognized by multiple Ors. Based on the limited experimental information, one might speculate that odorant discrimination may be the result of a combinatory code where different odorants stimulate unique sets of Ors and ORNs.

Functional Expression In Vivo. This strategy involves targeted gene expression in the sensory neurons of the olfactory epithelium followed by functional studies *in vivo*. Zhao et al. (1998) and Araneda et al. (2000) used a recombinant adenovirus to drive expression of a defined *Or* gene in an increased number of rat olfactory neurons. Electrophysiological recording showed that overexpression of a single gene led to greater sensitivity for a small subset of odorants (C7–C10 saturated aliphatic aldehydes). Zhao et al. (1998) demonstrated that Ors mediate a physiological response with some specificity. However, the window of specificity is somewhat broad because several compounds that possess some chemical similarities could activate the same receptor.

Functional studies have also been conducted in the nematode *C. elegans*, which offers a simple behavioral test based on attraction–repulsion according to the neuron were the Or is targeted. Such analysis led to the identification of the diacetyl receptor ODR-10 in *C. elegans* (Sengupta et al., 1996). Functional

expression of a mammalian *Or* using *C. elegans* as a reporter system has also been achieved (Milani et al., 2002). Transgenic nematodes expressing rat receptor I7 in targeted neurons showed modified odorant responsiveness during volatile attraction or avoidance behaviors. Thus, *C. elegans* appears to be a good *in vivo* system to test functional properties of Ors from different origins.

In addition, a new membrane protein was discovered in *C. elegans* (ODR-4), and this protein is thought to participate in membrane targeting of Or (Dwyer et al., 1998). Indeed, co-expression of ODR-4 with a rat Or in nonmature olfactory cells displayed perfect membrane targeting (Gimelbrant et al., 2001). ODR-4 homologs in vertebrates or insects have not yet been discovered.

Structure and Modeling. Our knowledge of Or structure–function relationships could be enhanced through the elucidation of Or three-dimensional structure. However, X-ray diffraction analyses of crystallized Or protein, and GPCR in general, is challenging because of the difficulties in obtaining large quantities of purified protein for crystallization. Combination of computer modeling and site-directed mutagenesis in Or could help in the identification of potential ligands and exploration of odorant binding sites. Currently, vertebrate protein modeling has been used to successfully identify receptor agonists and antagonists by virtual screening of compound libraries (Bissantz et al., 2003).

Initial Or Functional Studies in Insects

Clyne et al. (1999b) first showed that a mutation that alters the expression of a subset of *Or* genes alters the odorant specificity of a subset of *Drosophila* ORNs. Direct evidence for the involvement of one *Or* gene in olfaction came from two complementary and concomitant studies with *Drosophila* (Störtkuhl and Kettler, 2001; Wetzel et al., 2001). Using the GAL4/UAS system, an inducible targeted gene expression system widely used in *Drosophila*, Störtkhul and Kettler (2001) overexpressed the *Or43a* gene in the third antennal segment and tested for an increase in odor response *in vivo* by using electroantennography (EAG). Whereas *Or43a* is expressed in 15 neurons of wild-type fly antennae, overexpression led to approximately 1,200 neurons expressing *Or43a*. Overexpression conferred increased EAG responses to several structurally related compounds: cyclohexanol, cyclohexanone, benzaldehyde, and benzyl alcohol, all sharing a six carbon ring with a single polar group. Wetzel et al. (2001) expressed *Or43a* in *Xenopus* oocytes, a heterologous system. Using two-electrode voltage-clamp recording, they showed that the same four odorants from the *in vivo* study also activated the expressed receptor in the *Xenopus* system.

Although these studies provide direct evidence for Or43a function, they raise unexpected questions. In particular, can Or be activated without intervention of insect OBPs (expression in *Xenopus* oocytes) or in the presence of different OBPs from the one naturally expressed in the vicinity of the Or (overexpression)? It is

possible that unspecific binding protein(s) that could replace OBP function are present in these systems.

These studies conducted in *Drosophila* provide the first evidence for an *Or* gene function in insects, as well as for ligand determination. Recently, Dobritsa et al. (2003) investigated how the molecular and cellular maps of the *Drosophila* olfactory system are integrated by establishing the correspondence between individual neurons, odorant receptors, and odorants. Using either receptor substitution experiments in a mutant neuron or analysis of strains in which *Or* promoters were used to drive reporter genes, several *Or* genes were shown to confer response to particular odorants or were mapped to particular functional classes of neurons. In particular, *Or22a* maps to a neuron that responds to ethyl butyrate. Through a deletion mutant lacking *Or22a* and transgenic rescue analysis, Dobritsa et al. (2003) demonstrated that *Or22a* is required *in vivo* for response to this compound.

Similarly, Hallem et al. (2004b) have undertaken a systematic functional analysis of a variety of *DOr* genes that combines molecular and electrophysiological approaches. The determination of the odor spectrum of each Or allowed the authors to establish a receptor-to-neuron map of the *Drosophila* antennae by matching receptor spectra to defined ORN spectra. Thirty-one of the 32 *Dor* genes expressed in the antennae have been investigated. Receptors vary in their tuning breadth, and odorants vary in the number of receptors that they activate. In addition, excitation and inhibition, the two modes of olfactory signaling used by ORNs, are determined by the Or they express. Depending on the odorant, a single Or mediates both excitatory and inhibitory responses. Thus, Ors confer not only the odor response spectrum but also the response mode and the response dynamics upon the ORNs that express them (Hallem et al., 2004b). This study greatly advances our understanding of the molecular basis for odor coding in *Drosophila*.

Up to now, only one nondrosophilian Or has been assigned to a ligand. Hallem et al. (2004a) used *Drosophila* as an *in vivo* expression system for the *A. gambiae AgOr1*. *AgOr1* was expressed in *Drosophila* ORNs that normally express *Or22a* and *Or22b*, but these genes were deleted in the experimental construct. Responses to odors by the transformed neurons were assayed using single-cell electrophysiology. Female-specific AgOr1 is thought to participate in host-seeking behavior. Indeed, the functional study revealed that AgOr1 confers a strong response to 4-methylphenol, a component of human sweat (Hallem et al., 2004a).

Functional studies of insect Ors reveal broad odorant selectivity, as observed for vertebrate Ors (Malnic et al., 1999). This leads to a general concept of a combinatorial receptor code for odors with the following precept, verified at least in vertebrates: an Or from an individual neuron recognizes multiple odorants and, reciprocally, a single odorant is recognized by multiple receptors in different neurons. In the latter case, the multiple receptors have variable affinities for the ligand. Combination of specificity and tolerance, correlated with odorant concentration,

establishes a basal level of discrimination as well as the possibility to detect a wide range of various odorants, even with a low population of Ors.

SIGNAL TERMINATION, ADAPTATION, AND MODULATION

Signal reduction–inactivation and cessation are important components of olfactory perception. Different mechanisms may regulate agonist concentration. For example, biotransformation enzymes (see "General Olfactory Process") function to inactivate the signal (Figure 7A). In a recent example, a cytochrome P450 specific to the male antennae of the chafer beetle *Phylloperta diversa*, has been characterized as a potential pheromone-degrading enzyme (Maïbèche-Coisne et al., 2004). Adaptation is another process by which odorant perception depends on the previous experience of the ORNs. Thus, temporal information may be an important part of the chemosensory code. Reduction of receptor signaling is characterized at the molecular level by a process known as desensitization. It can affect the different steps of the transduction cascade or the GPCR itself. GPCR desensitization involves phosphorylation of the receptor by either protein kinases (PK) or G-protein-coupled serine–threonine receptor kinases (GRKs). While PKs are involved in slow desensitization processes, GRKs are responsible for rapid desensitization. GRKs phosphorylate the activated form of the receptor, and this in turn allows binding of an arrestin protein, which further uncouples the receptor from the signaling cascade (Figure 7B). Arrestins also trigger the endocytotic internalization of receptors, which is an integral component of GPCR resensitization in many systems (Figure 7B). For example, arrestins from *Drosophila* have been shown to be involved in visual signaling (Hyde et al., 1990; Smith et al., 1990). In addition to *Drosophila*, arrestins have been cloned in antennal tissues from *H. virescens* and from the migratory locust, *Locusta migratoria* (Raming et al., 1993b), and more recently from *A. gambiae* (Merrill et al., 2002, 2003). The functional significance of the arrestins has been investigated only in *Drosophila* (Merrill et al., 2002). Indeed, *Drosophila* visual arrestins appear to have a bimodal expression, in both photoreceptor and chemosensory neurons. Although their exact role is not yet understood, they are required for proper olfactory function, as arrestin mutants exhibit a decrease in the amplitudes of the electrophysiological responses to olfactory stimuli (Merrill et al., 2002).

Several factors have been shown to modulate the olfactory response of insects from a behavioral and electrophysiological point of view. These include prolonged exposure to plant volatiles (Stelinski et al., 2003), preexposure to sex pheromone (Anderson et al., 2003), circadian rhythms (Krishnan et al., 1999; Page and Koelling, 2003), or physiological states like mating. All of these effects suggest neuronal plasticity. However, the molecular mechanisms underlying these

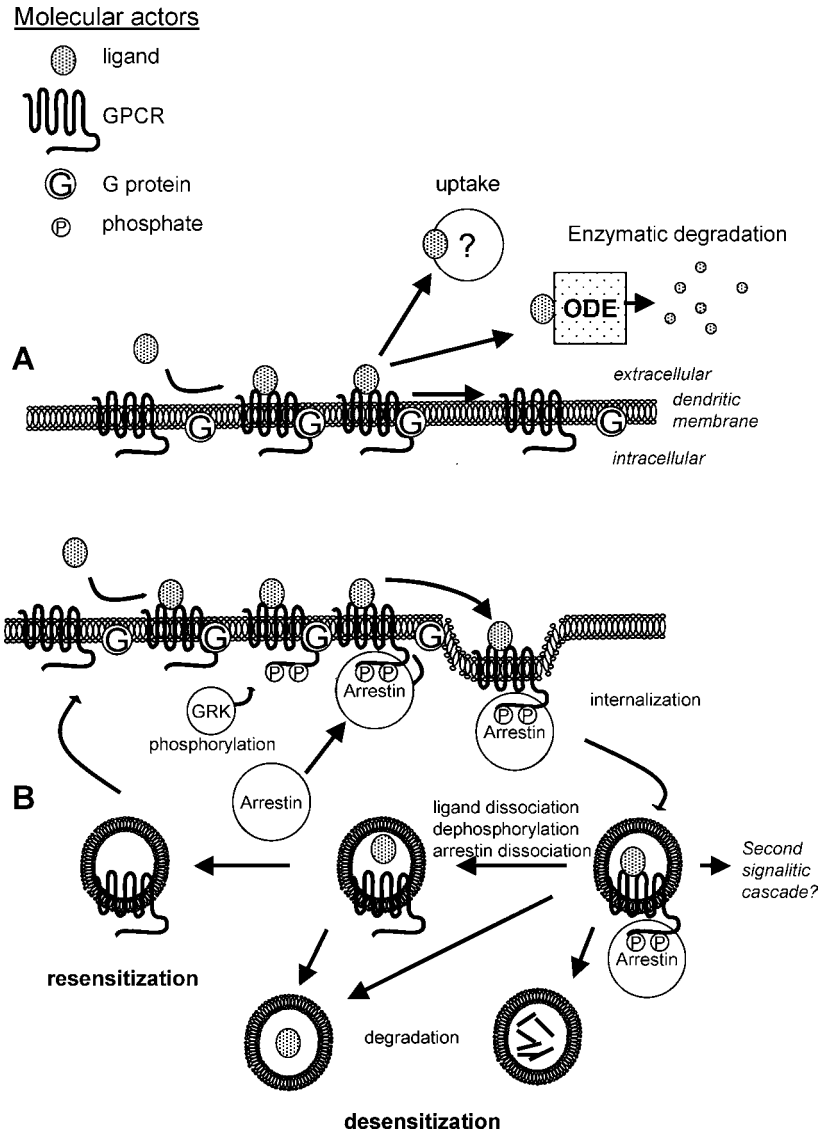

FIG. 7. Hypothetical mechanisms for regulation of agonist concentration in insect olfactory neurons. A: Signal inactivation could result from ligand uptake or enzymatic degradation by ODE (Odorant degrading enzyme). B: GPCR desensitization involves receptor phosphorylation by GRK (G-protein coupled receptor kinase), binding of an arrestin protein, internalization of the receptor, followed by receptor and/or ligand degradation or receptor resensitization.

phenomena are not yet understood. In particular, it is not known if regulation occurs at the peripheral and/or the central level of odorant signal integration. From a chemical ecological point of view, these phenomena are fundamental to understanding olfactory responses at the individual or population levels.

There is a potential role of Ors in olfactory sensitivity. Indeed, sensory experience and sensory activity regulate chemosensory receptor gene expression in *C. elegans* (Peckol et al., 2001). In addition, olfactory sensitivity has been shown to be down regulated after blood feeding in *A. gambiae* (Takken et al., 2001), which could be correlated with down-regulation of mRNA of *AgOr1* in this species (Fox et al., 2001). This *Or* displays female-specific olfactory tissue expression that may indicate a role in establishing host selection. This demonstrates that down-regulation of the expression of a specific gene may, in part, explain the observed decrease in host-seeking behavior, by modification of mosquito odorant response profile.

OTHER POSSIBLE FUNCTIONS FOR Ors

From a developmental standpoint, Ors have been proposed to play a crucial role in building the sensory map in the vertebrate olfactory bulb (Mombaerts et al., 1996; Wang et al., 1998; Strotmann et al., 2004). Specifically, they may determine axonal guidance of ORNs toward their glomerular targets. Indeed, all ORNs expressing the same *Or* converge to the same glomerulus, in vertebrates as well as invertebrates (see "Odor Coding: From Stereochemical Information to an Olfactory Sensory Map in the Brain"). However, *Drosophila Ors* seem to be expressed only after the establishment of synaptic connections (Vosshall et al., 2000). In addition, a recent study conducted in *Drosophila* showed that axonal targeting does not depend on normal *Or* expression (Dobritsa et al., 2003). Indeed, the ab3A neuron finds its normal glomerular target in a mutant that lacks the normally expressed Or in ab3A. Moreover, when other *Ors*, known to be expressed in ORNs that target a distinct glomerulus, were substituted for natural *Ors*, the axons were again observed to project to the normal glomerulus. Dobritsa et al. (2003) concluded that the ab3A neuron finds its glomerular targets through mechanisms independent of Or expression.

Alcedo and Kenyon (2004) have reported an unexpected role for Ors in the nematode *C. elegans* whereby specific subsets of both gustatory and olfactory neurons influence longevity. Some of these neurons inhibit longevity, whereas others promote it. Using RNA-mediated interference (RNAi, see "Future Perspectives, Opportunities, and Challenges of Insect Or Studies"), the authors observed that a decrease in mRNA level for GPCR str-2 in these chemosensory neurons extended life span. This suggests that an environmental cue (although yet unidentified) perceived through str-2 may influence life span. Although this surprising study is limited to *C. elegans*, the sensory system may influence longevity in

other organisms as well. Indeed, the *C. elegans* gustatory neuron influence on life span is likely to be mediated by the insulin/IGF-1 signaling pathway (Alcedo and Kenyon, 2004). This signaling has already been shown to extend life span in *Drosophila* (Tatar et al., 2001) and mice (Holzenberger et al., 2003). In addition, the smell of food has been reported to increase insulin levels in humans (Brand et al., 1982), although the effectors of this odor-stimulated pathway have not been yet deciphered.

FUTURE PERSPECTIVES, OPPORTUNITIES, AND CHALLENGES OF INSECT Or STUDIES

Or Sequence Identification and Development of Functional Studies. Since the completion of the *Drosophila* genome, insect genome sequencing is now growing rapidly. The first draft of the *Bombyx mori* genome has been published (Mita et al., 2004), and a draft of the *Apis mellifera* genome was released in January, 2004 (www.hgsc.bcm.tmc.edu/projects/honeybee/). Other insect genomes are on their way for sequencing; species soon to follow include *Drosophila pseudoobscura* (http://www.hgsc.bcm.tmc.edu/projects/drosophila/). This will no doubt lead to the identification of more Or candidates from a variety of species.

Functional studies, as well as ligand determination, will continue to be needed to confirm the role of the newly discovered Ors in odor detection and selection. From heterologous expression systems such as cell cultures, in particular insect cells, to *in vivo* gene expression modifications (overexpression, silencing), insects offer a wide array of genetic tools that can be combined with electrophysiological, pharmacological, and behavioral approaches. In particular, gene invalidation by double-stranded RNA interference (RNAi) allows suppression of gene expression in a sequence-specific manner, by targeting homologous mRNA degradation (for a review see Hammond et al., 2001). Specific gene silencing in targeted tissues allows elucidation of the biological role of the protein product. Although neurons appeared initially to be resistant to RNAi, effective RNAi has been obtained recently in adult *Drosophila* ORNs (Kalidas and Smith, 2002), providing a powerful tool to manipulate *Or* expression. Although *Drosophila* appears as the academic model with an abundance of molecular resources, considerable efforts have been made in developing similar tools in other insect species. Indeed, germline transformation protocols have been expanded to a wide range of species (Handler, 2001), including moths (Tamura et al., 2000) and mosquitoes (Grossman et al., 2001). Together with the development of gene expression modification tools, this will allow functional studies of insect Ors in agricultural pests or disease vectors.

With sequences in hand, the comparison of intra- and interspecific variants of a given Or may provide useful information about structure–activity relationships. Moreover, comparative studies of potential Ors from insects that use different

host–mate selection processes may provide information on their molecular basis. In addition, sex-specific expression in a given species may be relevant. For example, female-specific expression, observed for five *A. gambiae* Ors, could be especially relevant for disease transmission, which only occurs via females during the course of the blood meal. Male-specific Ors in moths may be implicated in response to female sex pheromones.

Identification of Pheromone Receptors. Among Ors, identification of pheromone receptors is a particular challenge, since pheromones mediate complex innate behaviors, most notably courtship and mating behavior. In mammals, pheromone receptors are expressed in a physically distinct sensory neuroepithelium from the main olfactory system: the vomeronasal organ. Only one of the candidate pheromone receptors has been proven to be functional in mice (Del Punta et al., 2002). These receptors show no sequence similarity to vertebrate Ors expressed in the olfactory epithelium, suggesting an independent evolutionary history. In insects, candidate pheromone receptors have been identified in *Drosophila* (Bray and Amrein, 2003) and in the moth *H. virescens* (Krieger et al., 2004). The *Drosophila* sex pheromone is composed of nonvolatile cuticular hydrocarbons, which may be detected by the male through the gustatory system. Bray and Amrein (2003) identified a putative gustatory receptor, Gr68a, which is expressed in chemosensory neurons of gustatory bristles in the forelegs. Using a molecular genetic approach that employs neuron inactivation (tetanus toxin) and gene invalidation (RNAi), in combination with a behavioral assay, they were able to show that Gr68a is an essential component of pheromone-driven courtship behavior in *Drosophila*. Inactivation of Gr68a-expressing neurons, as well as silencing of *Gr68a*, led to significant reduction in male courtship performance. It has been proposed that males use Gr68a to recognize female pheromone in the early steps of courtship, primarily through the tapping of female abdomen by male forelegs.

Potential use in Crop Protection or Animal and Human Health. Herbivorous insects are responsible for 25% of agricultural losses in the world and mosquitoes, as disease vectors, remain one of the leading causes of human mortality. For example, in Africa alone, malaria is responsible for more than 2 million deaths per year. As insects primarily use host volatiles to seek and select potential hosts, deciphering the molecular mechanisms of olfaction is essential for understanding odor discrimination and sensitivity that mediate host preference. This could allow improvement of already employed olfactory-based pest management strategies, as well as development of novel strategies.

Synergistic effects of different odorants, olfactory response modulation, ORN adaptation and desensitization, are known phenomena that could interfere with olfactory-based pest management tools such as mass trapping or mating disruption. In particular, the physiological state of the individuals appears important for the efficiency of such strategies. Indeed, after mating (Gadenne et al., 2001) or after feeding (Takken et al., 2001), insects undergo behavioral transitions where an

attractive cue can switch to an inactive or even repulsive one. A better knowledge of the molecular basis of olfactory sensitivity could assist in the improvement of these strategies.

Identification and functional characterization of molecular elements of the olfactory pathway, including Ors but also OBPs, SNMPs, GCs, and arrestins, will open the way toward the development of novel and innovative applications for controlling insects based on olfactory-mediated behaviors. Among them, targeted inhibition of the reception of a specific chemical compound, via Or engineering, would alter the blend ratio and lead to misperception of the chemical message. Experimental verification of predicted GPCRs is an essential step in pursuing these studies, and further exploration of GPCRs will facilitate the discovery of new pharmacological targets. Indeed, it is estimated that drugs that target GPCRs account for more than half of the medicines currently used, whatever the therapeutic domain (Galvez and Pin, 2003). In addition, the important role that Ors are likely to play in mediating host preference suggests that their study will likely contribute to the generation of new insect attractants or repellents for both crop protection and human health.

CONCLUDING REMARKS

From a fundamental and basic point of view, the mechanisms of odor recognition and the ensuing signal transduction seem to be identical or closely related in phylogenetically diverse species. This may reflect a striking evolutionary convergence towards a conserved organization of signaling pathways in olfactory systems. Insects are good models for studies of olfaction, as they offer not only a compartmentalized olfactory system, but also the possibility of using genetic engineering in combination with integrated physiological, pharmacological, and behavioral studies. However, as reflected in this review, we are still far from answering the question of how the olfactory system processes distinct olfactory cues to elicit appropriate behavioral responses. For instance, attractive or repulsive behaviors can be achieved from the same odorant stimulation depending on the species, the physiological state of the individual, or the concentration of the odorant.

Specificity and differential expression of Ors, along with OBPs, ODEs, or SNMPs, establishes unique functional phenotypes for different sensilla. In particular, while mammals express one or two OBPs in their olfactory system, insects express a diversity of OBPs in their antennae (e.g., 25 in *Drosophila*). On the contrary, insects express a relatively low number of Ors (62 DOrs, 79 AgOrs) (Hill et al., 2002; Robertson et al., 2003) when compared with vertebrates (1000 in mammals) or *C. elegans* (800). Although the biological significance of these observations is not known, it could be postulated that combinatorial expression of these different elements allows detection of a large number of odors. Because the

different actors may act in combination to trigger a specific response, studies of Ors should be undertaken to investigate the mechanisms in an integrative manner. Although many of these actors have been discovered and identified, their functional interaction leading to odor recognition is not yet clearly understood. The exact role of OBPs as the first line of discrimination has still not been demonstrated, there is no evidence about the exact nature of the Or agonist (odorant alone or the complex odorant–OBP), most of identified Ors are orphan receptors, and SNMP and GC functions are not yet understood.

The development of promising technologies, like *in vivo* functional imaging or behavioral genetic approaches, along with a growing understanding of the neuroanatomy of the system, will certainly in the near future match molecular structure with biological significance. Two invertebrate models, *D. melanogaster* and *C. elegans*, have proved to be useful in increasing our understanding of olfaction and olfactory-mediated behaviors, either as model animals to study their own receptors or as experimental systems to study *in vivo* heterologous Ors from a wide range of species. With the extension of genomic data and genetic tools, this information is rapidly being transferred to other insects, particularly those of medical or economical importance. This will undoubtedly result in great advances in understanding chemical communication mechanisms as well as in the future development of pest management strategies to reduce the negative effects of insects.

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REVIEW ARTICLE

GENOMIC HARDWIRING AND PHENOTYPIC PLASTICITY OF TERPENOID-BASED DEFENSES IN CONIFERS

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Abstract—Over evolutionary history, conifers have faced a myriad of threats from phloem- and xylem-feeding insects, defoliating insects, and fungal pathogens. Among the trees' defenses, terpenoids appear to play a major role by harming, disabling, deterring, repelling, or otherwise reducing the fitness of potential invaders. Each of the three classes of terpenoids in conifers, monoterpenes, sesquiterpenes, and diterpenes, are composed of a large number of representative compounds. In most cases, the presence of a particular terpenoid compound in the oleoresin or volatile emissions from a specific conifer can be accounted for by the expression of one of many committed terpene synthase (TPS) genes. However, while each TPS may produce one or a few major products, many produce a variety of minor products with relatively constant component ratios in the product blends. TPS genes exist in conifers in large and functionally diverse, yet monophyletic, gene families. Within these gene families, new biochemical functions of TPS appear to have evolved by gene duplication and changes in the amino acid sequence of the enzyme's active site. In addition, TPS genes may be differentially expressed prior to, during, and following attack by insects or pathogens. Thus, while the production of any particular terpenoid is hardwired into a conifer's genome, these trees have the capacity to change the mixture of terpenoids in oleoresin secretions and volatile

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emissions. Anatomical changes may also accompany induced terpenoid production, supplementing the plasticity of the molecular and biochemical events.

Key Words—Secondary metabolites, methyl jasmonate, traumatic resin ducts, volatile emissions, chemical defense, terpene synthase, conifer insect defense.

INTRODUCTION

The class Gymnospermae consists of four divisions: Ginkgophyta (one family, one genus), Gnetophyta (three families, three genera), Cycadophyta (three families, 11 genera), and Coniferophyta (seven families, ~61 genera) (van Gelderen and van Hoey Smith, 1996). Within the diverse Coniferophyta, four genera in the family Pinaceae stand out in terms of economic importance and dominance in the landscape. They are the true firs, *Abies* spp. (~50 species), the pines, *Pinus* spp. (70–100 species), the spruces, *Picea* spp. (~50 species), and Douglas-fir and its allies, *Pseudotsuga* spp. (5–20 species) (Vidakovié, 1991; van Gelderen and van Hoey Smith, 1996). Since their origins in the Carboniferous Period (~300 m.y.a.) (Schmidt and Schneider-Poetsch, 2002) and throughout their evolutionary history, conifers have been in contact with insects, which arose at least as early as the Silurian Period (~400 m.y.a.) (Engel and Grimaldi, 2004), and fungi. As a consequence, conifers have evolved sophisticated mechanisms of defense against these antagonists. One of the major defenses mounted against invading pests and pathogens is the secretion of copious oleoresin, comprised mainly of numerous 10-carbon monoterpenes, 15-carbon sesquiterpenes, and 20-carbon diterpenes, and their derivatives (Bohlmann and Croteau, 1999; Phillips and Croteau, 1999; Trapp and Croteau, 2001a; Langenheim, 2003) (Figure 1).

Terpenoid defenses provide both a physical and a chemical barrier against antagonists. Various oleoresin components in their fluid form are known to have toxic effects on insects and fungi when ingested or contacted by the antagonists (Smith, 1963; Raffa et al., 1985; Himejima et al., 1992; Werner, 1995). The volatile monoterpene and sesquiterpene components of the oleoresin can also act to alter insects' behavior before they feed (Reed et al., 1986; Byers, 1995; Seybold et al., 2000; Wallin and Raffa, 2000). The resin flow (Nebeker et al., 1993) may act to flush out invaders (Raffa and Berryman, 1982), and the non-volatile diterpene component that is left behind following the volatilization of the monoterpenes and sesquiterpenes acts to trap invading insects and pathogens in a crystalline matrix (Hodges et al., 1979).

The efficacy of terpenoid-based defense against a particular antagonist, like other chemical defenses (e.g., polyphenolics, proteinase inhibitors, etc.), depends on multiple factors. These include: (a) the speed at which the defense is mounted, (b) the timing of the defense in comparison to antagonist phenology, (c) the physical characteristics of the defensive secretion (oleoresin), (d) the precise compounds contained in the defensive secretion, (e) the bouquet of volatiles released

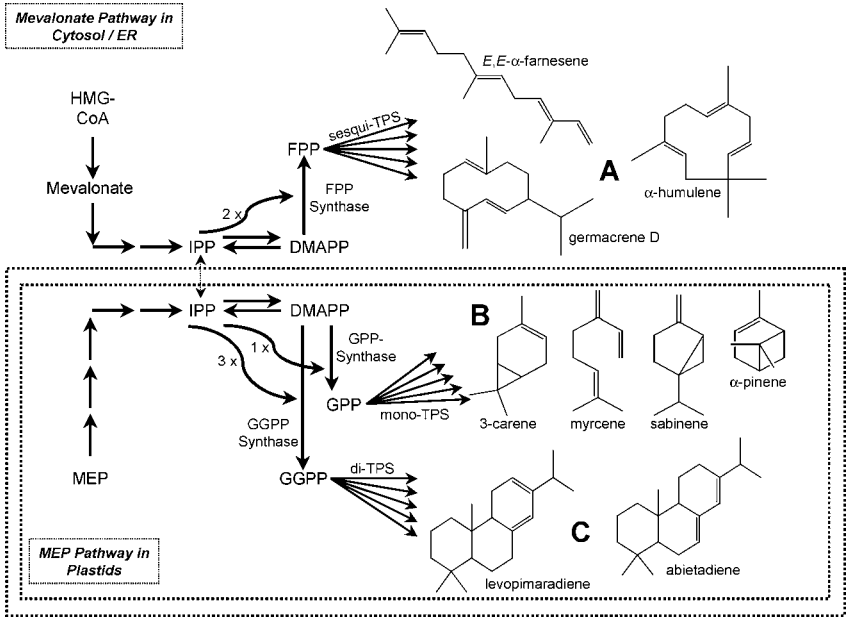

FIG. 1. Schematic diagram of the mevalonate (MVA) pathway in the cytosol/ER and the MEP pathway in the plastids of conifers. Fifteen-carbon sesquiterpenoids (A) (three representatives of which are shown) are produced by numerous sesquiterpene synthases in the late reactions of cytosolic MVA pathway. Ten-carbon monoterpenoids (B) and twenty-carbon diterpenoids (C) (four and two representatives of which are shown, respectively) are produced by numerous monoterpene synthases and diterpene synthases in the late reactions from intermediates of the plastidic MEP pathway. IPP and possibly other intermediates may be able to cross plastid membranes, leading to some interaction (crosstalk) between the two pathways. Abbreviations are as follows: MEP is 2C-methyl-D-erythritol 4-phosphate, HMG-CoA is 3-hydroxy-3-methylglutaryl Coenzyme A, IPP is isopentenyl diphosphate, DMAPP is dimethylallyl diphosphate, GPP is geranyl diphosphate, FPP is farnesyl diphosphate, GGPP is geranylgeranyl diphosphate, mono-TPS refers to the monoterpene synthases, sesqui-TPS refers to the sesquiterpene synthases, and di-TPS refers to the diterpene synthases.

from a wound or from adjacent areas, and (f) the ability of the plant to deliver the defensive secretions to the appropriate location. In the case of long-lived conifer trees, qualitative and quantitative variation, or phenotypic plasticity, and genetic adaptation of the multi-component terpenoid chemical defense also seems to be an important factor in long-term protection against potential insect herbivores or pathogens of much shorter generation times. While the production of each individual terpenoid is hardwired into a conifer's genome, the plasticity

required to mount effective defenses against a plethora of different antagonists is a result of a number of factors: (a) the evolution of a large number of functionally diverse terpene synthases (TPS) organized in multi-gene families, (b) the partitioning of terpenoid biosynthesis at the subcellular level, (c) the complex product profiles of many of the TPS that form terpenoids from simple precursors, (d) differential TPS gene expression in different tissues, and (e) induced TPS gene expression with differential spatial and temporal TPS expression profiles in the challenged tree. Other factors that increase the plasticity of the response against insects or pathogens are the signaling and regulation of the pathways leading to TPS gene expression and an astonishing capacity of conifers to differentiate new anatomical structures for terpenoid formation and resin secretion into traumatic resin ducts that allow accumulation of the products of the TPS enzymes. Our intention with this review is to outline the molecular, biochemical, and related histological and physiological components of the plasticity of conifer terpenoid defenses in order to allow the reader to further investigate this topic.

EVOLUTION AND BIOCHEMICAL FUNCTIONS OF CONIFER TERPENE SYNTHASES

Phylogenetic analyses allow the inference that conifer TPS are derived from a common ancestor because, based upon amino acid similarities, all conifer TPS characterized to date cluster together in one subfamily (i.e., subfamily TPS-d) of known plant TPS (Bohlmann et al., 1998b; Aubourg et al., 2002; Martin et al., 2004). Within the TPS-d subfamily, monoterpene synthase (mono-TPS), sesquiterpene synthase (sesqui-TPS), and diterpene synthase (di-TPS) genes mostly cluster separately, indicating separate evolutionary trajectories for each of these three biochemical classes of enzymes (Figure 2). Conifer sesqui-TPS of the TPS-d group apparently evolved independently several times from mono- and di-TPS genes in ancestors (Martin et al., 2004).

Conifers typically contain a large number of functional TPS that together produce a large number of products. For example, grand fir, *Abies grandis*, contains at least seven mono-TPS genes, three sesqui-TPS genes, and one di-TPS gene (Stofer Vogel et al., 1996; Bohlmann et al., 1997,1998a,b, 1999; Steele et al., 1998a,b). Similarly, Norway spruce, *Picea abies*, contains a large family of diverse TPS of which five mono-TPS genes, three sesqui-TPS genes, and two di-TPS genes have been functionally characterized to date (Fäldt et al., 2003; Martin et al., 2004). Three different mono-TPS genes and one sesqui-TPS gene have been characterized from loblolly pine, *Pinus taeda* (Phillips et al., 2003). These numbers are a gross underestimate of the true number of TPS genes in the large genomes typical of conifer species (estimated 20–40 Gb), considering that more than 30 TPS genes were found in the sequenced *Arabidopsis thaliana* genome

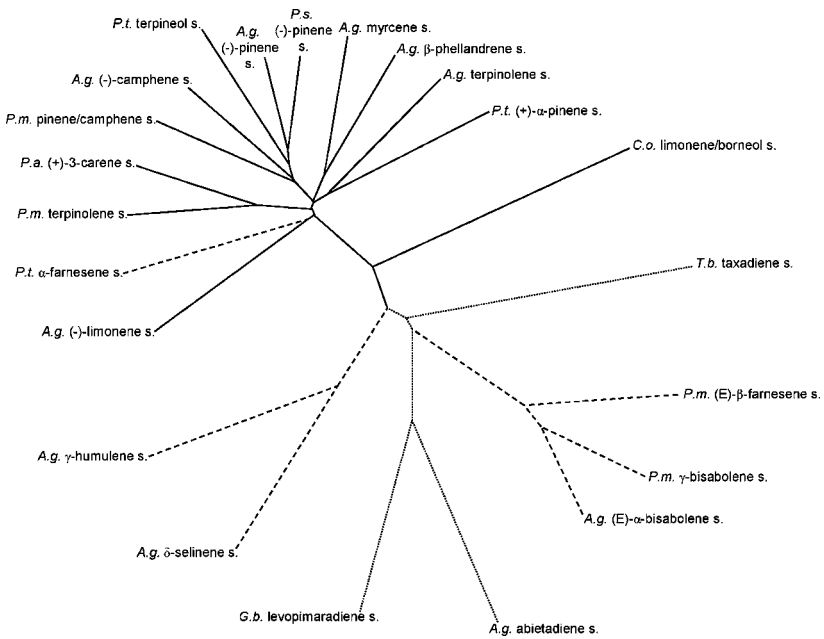

FIG. 2. Phylogenetic tree of representative terpenoid synthases (TPS) in the TPS-d subfamily. Solid lines (—) show branches containing monoterpene synthases, dashed lines (—) show branches containing sesquiterpene synthases, and dotted lines (. . .) show branches containing diterpene synthases. The three different groups mainly cluster separately, though there are exceptions. Taxon abbreviations are as follows: *A.g.* is *A. grandis*; *C.o.* is *Chamaecyparis obtusa*; *G.b.* is *Ginkgo biloba*; *P.a.* is *P. abies*; *P.s.* is *P. sitchensis*; *P.m.* is *P. menziesii*; *P.t.* is *P. taeda*; and *T.b.*is *Taxus brevifolia*. Most amino acid sequences were obtained from GenBank except for those for *P. menziesii*, which were from unpublished data from our lab. The alignment was completed with ClustalX (Thompson et al., 1997) and the tree was visualized with TreeView (Page, 1996).

of ca. 125 Mb (Aubourg et al., 2002). A survey of more than 90,000 expressed sequence tags (ESTs) developed as part of a Sitka spruce, *Picea sitchensis*, and white spruce, *Picea glauca*, genomics project (http://treenomix.com) suggests that the number of TPS genes in a spruce genome is much larger than the 30 TPS genes identified in the *Arabidopsis* genome.

The common origin of all conifer TPS genes (Bohlmann et al., 1998b, 2000; Trapp and Croteau, 2001b; Martin et al., 2004), in conjunction with the large number of TPS genes in conifer genomes, suggest that families of functionally diverse TPS genes arose from events of gene duplication, gene mutations, and functional diversification during the evolution of conifers. This pattern of TPS evolution is also becoming apparent from genome-wide analysis of TPS in

A. thaliana (Aubourg et al., 2002), and is supported by results from functional characterization and comparative analysis of TPS in *P. abies* (Martin et al., 2004). The fact that the three classes of conifer TPS genes (mono-TPS, sesqui-TPS, and di-TPS) each cluster within the TPS-d group further indicates that a hypothetical ancestral conifer had at least one of each class of genes before the conifer group radiated into the genera and species that we see today. Analysis of TPS genes in more species in the principal genera will test this evolutionary hypothesis. Considering the role of terpenoids in defense against insects and pathogens, it is also likely that co-evolutionary processes (Thompson, 1994) have shaped the array of TPS genes that we find in conifers.

The TPS-mediated reactions, as well as earlier steps of terpenoid biosynthesis, are partitioned in subcellular compartments (Croteau et al., 2000). Specifically, monoterpenoids and diterpenoids are produced in the plastids from five-carbon products of the methylerythritol phosphate (MEP) pathway, whereas sesquiterpenoids are produced in the cytoplasm from products of the mevalonate (MVA) pathway (Figure 1) (Croteau et al., 2000). Our knowledge of the MEP and MVA pathways in plants is largely based on studies with angiosperms, although mining of the rapidly growing spruce cDNA EST databases (http://treenomix.com) has identified almost all the genes of both pathways as expressed transcripts in conifers (Bohlmann et al., unpublished data). The subcellular partitioning of the two terpenoid biosynthesis pathways (Figure 1) could allow for the regulation of some terpenoid biosynthesis independently of others, although some crosstalk between the two pathways occurs (Laule et al., 2003).

Following the formation of the five-carbon intermediates, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), via the MEP and MVA pathways, terpenoid formation proceeds in the plastids and the cytosol with the activity of prenyldiphosphate synthases that generate the substrates for TPS enzymes (Figure 1). In the plastids, the formation of geranyl diphosphate (GPP) from one molecule of DMAPP and one molecule of IPP by GPP synthase is followed by the activity of mono-TPS that convert GPP to monoterpenoids. Diterpene synthesis, in the plastids as well, proceeds via the activity of geranylgeranyl diphosphate (GGPP) synthase, which makes GGPP from one molecule of DMAPP and three molecules of IPP, followed by the activity of di-TPS that convert GGPP to diterpenoids. To date, GPP synthase and GGPP synthase genes, their functions, and enzyme subunit organizations as homodimers have been described in the literature only for one member of the Pinaceae, grand fir, *A. grandis* (Burke and Croteau, 2002a,b). In the cytoplasm, farnesyl diphosphate (FPP) synthase converts one molecule of DMAPP and two molecules of IPP into FPP. Sesqui-TPS then catalyze the reaction from FPP to 15-carbon sesquiterpenes. Sequences for transcripts with high homology to *A. grandis* GPP synthase, GGPP synthase, and angiosperm FPP synthases are abundantly present and available for functional characterization in the spruce EST databases (http://treenomix.com).

The activities of the various TPS enzymes are also localized to the plastids or the cytoplasm (Bohlmann et al., 1998b; Croteau et al., 2000). Following the synthesis of each class of terpenoid, other enzymes may oxygenate them or add other functional groups, increasing the structural complexity of the final terpenoid products, Although secondary modifications of some terpenoids, such as the stepwise oxidations of the diterpene hydrocarbon abietadiene to abietic acid, are important for the physical, chemical, and biological properties of oleoresin defense secretions, little is known about the genes and enzymes of secondary modification of the primary terpenoid products in conifers (Funk and Croteau, 1994). Only recently has it been possible to clone and functionally identify the first cytochrome P450-dependent monooxygenase enzymes of diterpene resin acid formation from *P. taeda* (Ro and Bohlmann, unpublished data).

In contrast, a great deal of detailed information is available on the biochemical functions of known conifer TPS genes (Stofer Vogel et al., 1996; Bohlmann et al., 1997, 1998a, 1999; Steele et al., 1998a; Peters et al., 2000, 2001, 2003; Ravn et al., 2000, 2002; Peters and Croteau, 2002a,b; Fäldt et al., 2003; Phillips et al., 2003; Martin et al., 2004). The similarity in amino acid sequence among TPS enzymes (Figures 3 and 4) suggests similarity in catalytic function within each of the three classes of conifer TPS. While members of each class use the same substrate (GPP, FPP, or GGPP for mono-, sesqui- and di-TPS, respectively), the large number of TPS enzymes in any given conifer and the specific differences in the catalytic activities of each help to produce the enormous array of different terpenoids in conifer oleoresin. In addition, although a single TPS may produce just one terpenoid in substantial quantity (e.g., Bohlmann et al., 1997,1998a; Martin et al., 2004), many TPS produce significant amounts of numerous products in the same class in fairly consistent ratios (e.g., Steele et al., 1998a; Bohlmann et al., 1999; Peters et al., 2000; Fäldt et al., 2003; Martin et al., 2004). Reaction schemes have been proposed to explain the rich diversity of products from single conifer TPS enzymes acting on single substrates for mono-, sesqui-, and di-TPS (e.g., Steele et al., 1998a; Bohlmann et al., 1999; Fäldt et al., 2003; Martin et al., 2004). Specific examples of multiple product conifer TPS include two multiple product mono-TPS that were cloned and functionally characterized from Douglas-fir, *Pseudotsuga menziesii* (Huber et al., unpublished data). The first mono-TPS, a terpinolene synthase, also produces α- and γ-terpinene, α- and β-pinene, limonene, sabinene, myrcene, 3-carene, and trace amounts of camphene and α-thujene. The second, an α-pinene/camphene synthase also produces limonene, 3-carene, and β-pinene. Sesquiterpene synthases have been characterized in *A. grandis* and in *P. abies* that produce more than 10 different products each (Steele et al., 1998a; Martin et al., 2004). Characterization of TPS genes in *P. abies* identified a pair of di-TPS of 90% amino acid identity, one of which forms a single diterpene product, isopimara-7,15-diene, and the other that produces four different diterpenes, levopimaradiene, abietadiene, neoabietadiene, and palustradiene (Martin et al., 2004).

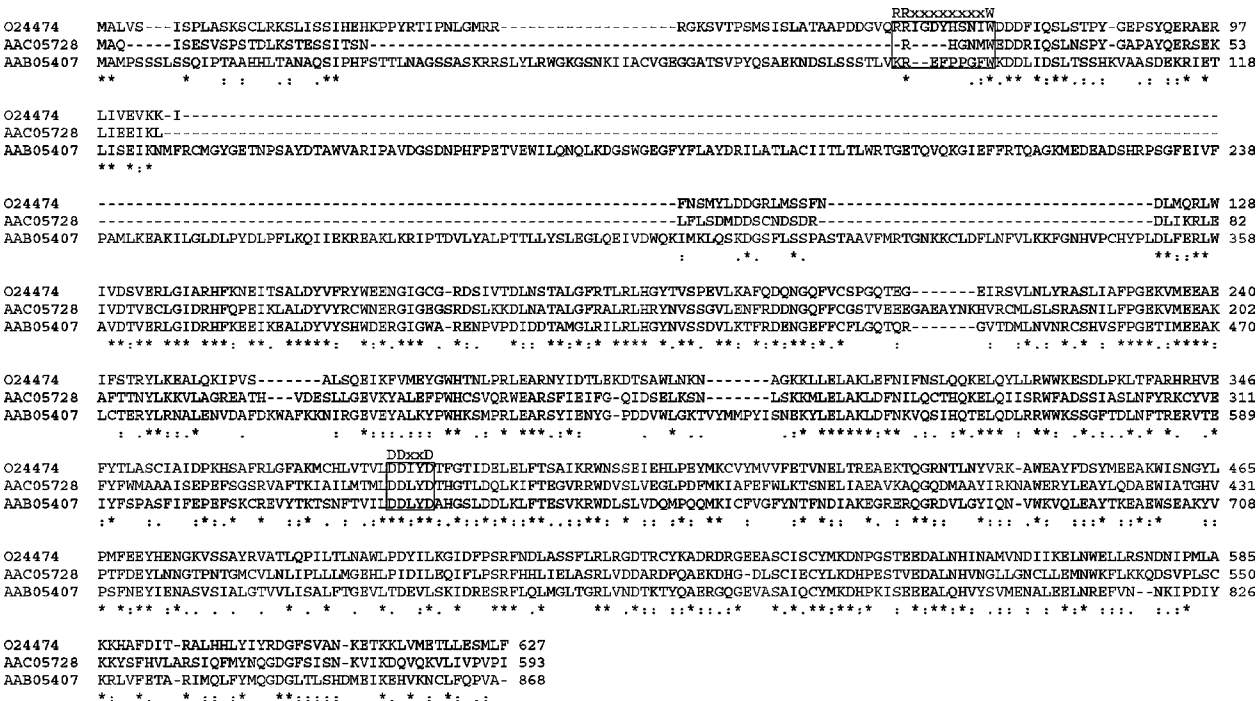

FIG. 3. Amino acid sequence alignment of a monoterpene synthase (myrcene synthase; GenBank accession O24474), a sesquiterpene synthase (γ-humulene synthase; GenBank accession AAC05728), and a diterpene synthase (abietadiene cyclase; GenBank accession AAB05407) from grand fir, *A. grandis*. Identical residues in all three sequences are marked by "*", conserved substitutions by ":" and semi-conserved substitutions by ".". Marked in the alignment are the DDxxD active site motif, associated with binding of divalent metal cofactors, and the RRX8W motif, associated with initial isomerization steps. Conifer diterpene synthases are usually about 210 amino acid residues longer than either monoterpene synthases or sesquiterpene synthases. Throughout the rest of the alignment, however, similarity is high among sequences of the three biochemical classes. The alignment was completed with ClustalW (Thompson et al., 1994) and JalView (Clamp et al., 2004).

```
AAB70707      MALLSITPLVSRSCL-----SSSHEIKALRRTIPTLGICRPGKSVAHSIN 45
O24475        MALVSTAPLASKSCLHKSLISSTHELKALSRTIPALGMSRRGKSITPSIS 50
              ***:* :**.*:***     **:**:*** ****:**:.* ***:: **.

                          RRxxxxxxxxW
AAB70707      MCLTSVASTDSVQRRVGNYHSNLWDDDFIQSLISTPYGAPDYRERADRLI 95
O24475        MSSTTVVTDDGVRRRMGDFHSNLWDDDVIQSLP-TAYEEKSYLERAEKLI 99
              *. *:*.: *.*:**:*::********.****  *.*   .* ***::**

AAB70707      GEVKDIMFNFKSLEDG-----GNDLLQRLLLVDDVERLGIDRHFKKEIKT 140
O24475        GEVKN-MFNSMSLEDGELMSPLNDLIQRLWIVDSLERLGIHRHFKDEIKS 148
              ****: ***  *****      ***:*** :**.:*****.****.***:

AAB70707      ALDYVNSYWNEKGIGCGRESVVTDLNSTALGLRTLRLHGYTVSSDVLNVF 190
O24475        ALDYVYSYWGENGIGCGRESVVTDLNSTALGLRTLRLHGYPVSSDVFKAF 198
              ***** ***.*:****************************.*****::.*

AAB70707      KDKNGQFSSTANIQIEGEIRGVLNLFRASLVAFPGEKVMDEAETFSTKYL 240
O24475        KGQNGQFSCSENIQTDEEIRGVLNLFRASLIAFPGEKIMDEAEIFSTKYL 248
              *.:*****.: *** : *************:******:***** ******

AAB70707      REALQKIPASSILSLEIRDVLEYGWHTNLPRLEARNYMDVFGQHTKNKN- 289
O24475        KEALQKIPVSS-LSREIGDVLEYGWHTYLPRLEARNYIQVFGQDTENTKS 297
              :*******.** ** ** ********* *********::****.*:*.:

AAB70707      --AAEKLLELAKLEFNIFHSLQERELKHVSRWWKDSGSPEMTFCRHRHVE 337
O24475        YVKSKKLLELAKLEFNIFQSLQKRELESLVRWWKESGFPEMTFCRHRHVE 347
              ::*************:***:***: : ****:** ************

                                          DDxxD
AAB70707      YYALASCIAFEPQHSGFRLGFTKMSHLITVLDDMYDVFGTVDELELFTAT 387
O24475        YYTLASCIAFEPQHSGFRLGFAKTCHLITVLDDMYDTFGTVDELELFTAT 397
              **:******************:* .***********.*************

AAB70707      IKRWDPSAMECLPEYMKGVYMMVYHTVNEMARVAEKAQGRDTLNYARQAW 437
O24475        MKRWDPSSIDCLPEYMKGVYIAVYDTVNEMAREAEEAQGRDTLTYAREAW 447
              :******:::**********: **.******* **:*******.***:**

AAB70707      EACFDSYMQEAKWIATGYLPTFEEYLENGKVSSAHRPCALQPILTLDIPF 487
O24475        EAYIDSYMQEARWIATGYLPSFDEYYENGKVSCGHRISALQPILTMDIPF 497
              ** :*******:********:*:** ******..** .*******:****

AAB70707      PDHILKEVDFPSKLNDLICIILRLRGDTRCYKADRARGEEASSISCYMKD 537
O24475        PDHILKEVDFPSKLNDLACAILRLRGDTRCYKADRARGEEASSISCYMKD 547
              ***************** * ****************************

AAB70707      NPGLTEEDALNHINFMIRDAIRELNWELLKPDNSVPITSKKHAFDISRVW 587
O24475        NPGVSEEDALDHINAMISDVIKGLNWELLKPDINVPISAKKHAFDIARAF 597
              ***::*****:*** ** *.*: ********* .***::*******:*.:

AAB70707      HHGYRYRDGYSFANVETKSLVMRTVIEPVPL 618
O24475        HYGYKYRDGYSVANVETKSLVTRTLLESVPL 628
              *:**:******.********* **::*.***
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FIG. 4. Amino acid sequence alignment of two monoterpene synthases [(−)-camphene synthase, GenBank accession AAB70707; and (−)-pinene synthase, GenBank accession O24475] from grand fir, *A. grandis*. Identical residues in the two sequences are marked by "*", conserved substitutions by ":" and semi-conserved substitutions by ".". Marked in the alignment are the DDxxD motif and the RRX8W motif. The sequence similarity is high, making it impossible within a class of terpene synthases to ascertain the catalytic function of an enzyme from only its amino acid sequence. It is necessary to express newly discovered terpene synthases and perform assays with the appropriate substrate to fully characterize the enzyme. The alignment was completed with ClustalW (Thompson et al., 1994) and JalView (Clamp et al., 2004).

This brief review of the molecular and biochemical literature on terpenoid formation in conifers reveals that the many products of conifer terpenoid pathways can be seen as the expression of a genomic blueprint of the various portions of the terpenoid biosynthesis pathways, with multigene families of functionally diverse TPS as the core generators of the chemical diversity and plasticity of conifer terpenoid defense. Comparatively little is known about the molecular genetics and biochemistry of earlier and later steps in the terpenoid pathways in conifers (MEP, MVA pathways, prenyltransferases, and cytochrome P450 enzymes), and their contribution to genetic and biochemical control of terpenoid defense phenotypes. The many genes now available from large-scale conifer genome projects will accelerate functional characterization of these early and late pathway steps in the near future. The expression of terpenoid defense genes in conifers is differentially controlled both by endogenous and exogenous factors (see below), which shape the phenotypic plasticity of terpenoid profiles in conifer defense.

PRODUCTION AND DELIVERY OF TERPENOIDS DURING DEFENSE IN CONIFER STEM TISSUES

The molecular genetics and biochemistry of conifer terpenoid biosynthesis play an overriding role in the variable quantity and quality of terpenoids that a tree can produce. The plasticity of multigene-based terpenoid defense can be considered an important adaptive trait of long-lived conifers that interact on several trophic levels with faster evolving insects and microorganisms. However, regulation at the physiological, cellular, and anatomical levels in response to an insect infestation or a pathogen inoculation, and the associated ability of the tree to allocate terpenoids with appropriate spatial and temporal patterns for effective defense are also important in the flexibility of a conifer's terpenoid defense (Martin et al., 2002, 2003; Hudgins and Franceschi, 2004; Hudgins et al., 2004). Because conifers are typically long-lived and large organisms, an understanding of physiological aspects related to defense against insects and pathogens is not always easy to develop. Vincent Franceschi, Erik Christiansen and co-workers, and our research group, however, have developed systems to study aspects of conifer defenses, including terpenoid and phenolic-based defenses, at several levels, from anatomy and histology to physiology, biochemistry, and genomics in *Picea* spp.

One particularly well-studied system is the response of *Picea* spp. to the white pine weevil, *Pissodes strobi* (Alfaro et al., 2002). After overwintering in the duff, adult weevils climb the stem of small ($\sim$1.5–10 m) trees (Turnquist and Alfaro, 1996). When they reach the terminal shoot, they puncture the bark to feed and, after mating, to lay eggs. The larvae hatch and feed within the phloem, cambium, and the youngest outer xylem by burrowing downward from the site of oviposition until they complete their larval and pupal stages and then emerge as adults. The larval feeding takes a large toll on the tree by destroying the terminal

leader and forcing a nearby lateral branch to assume apical dominance in the following year. Repeated infestations by the weevil over a number of years reduce height growth, create infection courts for decay fungi, dramatically reduce wood quality, and increase the risk for a tree to be overgrown by competing vegetation. In response to weevil feeding or oviposition, extensive changes occur in the resin duct anatomy of the affected spruce leader, which normally contains large resin ducts in the bark, but only few, if any, constitutive axial resin ducts in the xylem. Axial traumatic resin ducts, induced by weevil feeding or oviposition, form rapidly in the newly developing xylem where they persist for several months or years (Alfaro, 1995; Byun McKay et al., 2003). The ducts begin to form quickly following early contact with the feeding or ovipositing insect, and it is thought that the rapid formation of ducts near the developing larvae allows the tree to deliver a payload of terpenoids directly to the site of insect residence and feeding. Simulation of insect attack on spruce or inoculation with fungi, often associated with insects, using drill wounding or treatment with an octadecanoid-derived plant defense elicitor, methyl jasmonate (MeJA) (Figure 5), causes similar traumatic resin duct formation in the developing xylem in *P. glauca*, *P. sitchensis*, and *P. abies* (Tomlin et al., 1998; Nagy et al., 2000; Franceschi et al., 2002; Martin et al., 2002, Byun McKay et al., 2003; Miller et al., 2005). Traumatic resin duct formation is also induced by MeJA or ethylene treatment in several other members of the pine family (Hudgins et al., 2003, 2004; Hudgins and Franceschi, 2004; Huber et al., 2005).

Mimicked insect attack of young spruce trees by using MeJA treatment has proven to be a powerful tool in experiments to decipher, in detail, the complex physiological and anatomical changes related to induced terpenoid accumulation, increased biochemical activities of terpenoid pathways, and differential TPS gene expression associated with traumatic resin duct formation in spruce stem tissues.

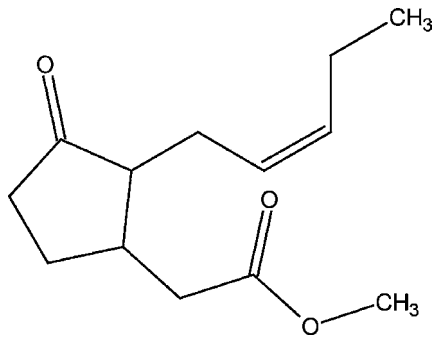

FIG. 5. General structure of methyl jasmonate (MeJA) (Baldwin, 1999). The enantiomeric composition of the biologically active form and endogenous octadecanoids in conifers have not been determined.

Similarly, treatment of spruce trees with MeJA also allows a detailed analysis of induced terpenoid volatiles emitted from spruce needles and of the biochemical processes involved in foliage (Martin et al., 2003). Topical applications of MeJA induce traumatic resin canal formation in species of spruce (Franceschi et al., 2002; Martin et al., 2002; Byun McKay et al., 2003) and other conifers (Hudgins et al., 2003, 2004). Some conifers, however, do not produce traumatic resin ducts upon application of MeJA, but instead either exhibit other histological modifications or no observable changes at all (Hudgins et al., 2003, 2004). MeJA treatment of *P. abies* saplings leads to rapid cell differentiation of traumatic resin ducts *in lieu* of regular xylem tracheids in the youngest developing xylem layers. The new cell structures appear next to the active cambium within a few days after treatment of trees with MeJA (Martin et al., 2002). This process of resin canal differentiation involves a re-direction of the normal developmental program of cambium cells in response to the octadecanoid signal. It is possible that other plant signal molecules are involved in the reprogramming of insect-induced resin canal differentiation downstream or upstream of the octadecanoid pathway. In addition to MeJA, a role for ethylene in the induction of traumatic resin ducts, polyphenolic parenchyma cells, and sclereid lignification in *P. menziesii* has recently been shown (Hudgins and Franceschi, 2004).

MeJA also has profound effects on TPS gene expression and enzyme activities associated with traumatic resinosis in *P. abies*. Transcripts associated with mono- and di-TPS genes increase rapidly in sapling stems following the topical application of 0.01% MeJA (Fäldt et al., 2003). For both classes of TPS genes, the peak in transcript levels occurs about 2 days after MeJA application and declines at day 4. Mono-TPS transcript levels remain slightly elevated even 16 days after MeJA treatment, whereas di-TPS transcript levels decline to levels similar to those prior to MeJA treatment (Fäldt et al., 2003). The increase in TPS mRNA following MeJA application is followed by increased TPS enzyme activities and changes in terpenoid content in *P. abies* stem tissues (Martin et al., 2002).

Monoterpenoids and diterpenoids are not constitutively present in large amounts in the xylem of *P. abies* stems, but they build-up rapidly in xylem of MeJA-treated saplings, and there is also a substantial increase in terpenoids in bark tissues, which are already constitutively rich in resin terpenoids (Martin et al., 2002). The same study showed that the overall enzyme activities of mono- and di-TPS mirrored the terpenoid build-up. Thus, MeJA induces rapid and long-lasting effects in spruce stem tissues including the induction of mono- and di-TPS gene expression, an increase in related enzyme activities (e.g., prenyl diphosphate synthases), an accumulation of induced resin terpenoids, and concurrent *de novo* formation of traumatic resin ducts (Martin et al., 2002; Fäldt et al., 2003). This is consistent with a rapid defense response to a real insect infestation followed by maintenance of terpenoid defense during a growing season. Similar to the induced expression of TPS genes in *P. abies* saplings in response to MeJA, TPS

gene transcripts also accumulate after real insect feeding by white pine weevil and after drill wounding of *P. sitchensis* (Byun McKay et al., 2003). Mono-TPS gene transcripts increase within one day of initial contact with weevils in the terminal growth of lateral branches of *P. sitchensis*, and peak at about 2–4 days. At 7 days, transcript levels are still somewhat elevated in lateral branches. Drill wounding of lateral branches and leaders give similar results. Probing of TPS gene expression on single trees of two genotypes showed that transcript levels of two mono-TPS genes, including a *P. sitchensis* (−)-α-pinene/(−)-β-pinene synthase, increase within 12 hr of drill wounding (Byun McKay et al., 2003). Recent studies with *P. sitchensis* saplings showed some differential, MeJA- and weevil-induced gene expression among 11 different mono-, sesqui- and di-TPS (Miller et al., 2005), suggesting some independent regulation of members of the TPS gene family in response to insect feeding.

Fungal inoculation of conifers can have effects on terpenoid content of resin similar to MeJA-treatment, suggesting the involvement of similar or identical genes. However, to our knowledge, no investigations into the molecular genetic foundation of this phenomenon have been published. Furthermore, microarray gene expression analysis of several thousand *P. sitchensis* genes in response to feeding by *P. strobi*, by the western spruce budworm, *Choristoneura occidentalis*, mechanical wounding, or MeJA treatment, have revealed large-scale changes of gene expression in *P. sitchensis*, with partially overlapping gene expression profiles common to all treatments (Ralph and Bohlmann, unpublished results; http://treenomix.com). Such microarray analysis of the transcriptome of conifers in response to real and simulated insect attack is not only leading to the discovery of metabolic pathways, such as terpenoid and phenolic pathways associated with defense in *P. sitchensis*, but also allowing the discovery of signaling cascades and transcription factors that can orchestrate complex, multi-gene defense responses.

METHYL JASMONATE- AND INSECT-INDUCED VOLATILE EMISSIONS

Dramatic changes in terpenoid biochemistry are also induced in the foliage of *P. abies* and *P. sitchensis*, in response to treatment with MeJA (Martin et al., 2003; Miller et al., 2005). While the build-up of terpenoids in foliage following MeJA application is not nearly as strong as in bark and wood, MeJA causes a large increase in the emission of volatile monoterpenoids and sesquiterpenoids. Measurements of the headspace around MeJA-treated *P. abies* and *P. sitchensis* saplings reveals not only an increase in terpenoid release from foliage, but also a major shift from emission of monoterpene hydrocarbons towards emission of sesquiterpenoids and oxygenated monoterpenoids, specifically farnesene, bisabolene, and linalool (Martin et al., 2003; Miller et al., 2005). Many angiosperms are known to release volatiles (in many cases terpenoids) in response to herbivory by

insects. These volatiles often function in the attraction of insect natural enemies of the herbivores (Takabayashi and Dicke, 1996; Paré and Tumlinson, 1997a,b, 1999; Dicke and Vet, 1999; Kessler and Baldwin, 2001, 2002; Arimura et al., 2004). Recently, it has also been shown that volatiles are induced from conifers upon herbivory and egg deposition in needles (Litvak and Monson, 1998; Hilker et al., 2002), and it is possible that many of these volatiles are emitted constitutively from insect-damaged resin storage sites in needles. However, simulated insect attack by MeJA treatment of *P. abies* and *P. sitchensis* (Martin et al., 2003; Miller et al., 2005) demonstrated that the induced emission of linalool and sesquiterpenoids from foliage is the result of rapidly induced up-regulation of foliar TPS gene expression and increased enzyme activities of a few monoterpene synthases and sesquiterpene synthases. Moreover, detailed qualitative analysis of temporal patterns of volatile profiles of *P. sitchensis* infested with *P. strobi* showed new emissions of linalool that cannot be explained simply by release from preformed resin terpenoids, but are likely derived from insect-induced *de novo* formation and induced release into the headspace (Miller et al., 2005).

The specific TPS gene expression and enzyme activities that are up-regulated in foliage of *P. abies* and *P. sitchensis* upon treatment with MeJA differ from those that are up-regulated after MeJA-treatment and insect attack in stems, supporting the concept of differential organ-specific regulation of terpenoid defenses in *Picea* spp.

SIGNALS INVOLVED IN INDUCED RESIN TERPENOID DEFENSES

The similarity in events of terpenoid responses and related anatomical defenses following insect feeding, simulated wounding, and MeJA application suggests that octadecanoids may play some role in terpenoid-based defense in conifers, specifically as signals leading to traumatic resinosis and terpenoid emissions. While MeJA or other octadecanoid compounds still remain to be detected as endogenous defense signals in *Picea* spp., we have recently identified many of the known octadecanoid pathway genes as transcripts in our *Picea* EST databse (http://treenomix.com). Increased mRNA levels of key steps of the octadecanoid pathway were found in response to both insect attack by *P. strobi* and in response to MeJA-treatment when the transcriptome of *P. sitchensis* was analyzed using microarrays (Ralph and Bohlmann, unpublished) and verified by Northern hybridizations (Miller et al., 2005). Future work will target additional signaling events and transcription factors that control changes in gene expression, terpenoid accumulation and emission, and anatomical changes in xylem resin duct development. Further, there are possible interactions of these factors with signaling networks, in which ethylene (Hudgins and Franceschi, 2004), auxins, or other signal compounds likely play a role.

GENOMIC APPROACHES TO CONIFER DEFENSE

Further work is needed in conifers to understand the orchestration of complex insect- or pathogen-induced defense genes and protein expression with the resulting chemical and anatomical defenses. Current genomics approaches in our laboratory use microarray transcriptome analyses and 2DE/LC-MS-MS-proteome analyses of trees challenged by insects, pathogens, or elicitors, as well as metabolite profiling of these trees (http://treenomix.com). These approaches allow for the simultaneous profiling of hundreds or thousands of different gene transcript, protein, and metabolite species, requiring careful statistical and bioinformatics analysis of large data-sets, which is now possible due to recent developments in conifer genomics. Genomic resources are also being developed in parallel for conifer-insect pests, specifically for bark beetles (Eigenheer et al., 2003; Tittiger, 2004), western spruce budworm, *C. occidentalis* (Qili Feng and Basil Arif, personal communication), and for conifer pathogens, specifically bark-beetle associated blue-stain fungi (ongoing research in our laboratory). Analysis of large data sets obtained by genomics approaches to conifer defense can result in the *in silico* reconstruction of signaling and metabolic pathway networks under constitutive and insect-induced conditions. Selected components of such signaling and pathway maps of insect-induced processes can then be tested functionally by using a combination of biochemical and genetic methods, that include the modification of target gene expression. Selected candidate genes can be further explored as genetic markers for association with insect-defense or resistance in natural or breeding populations. The simultaneous profiling of gene expression in conifers and their pests or pathogens may also lead to identification of key elements in the interacting genomes of these organisms.

SUMMARY AND FUTURE DIRECTIONS

The genomic, molecular, and biochemical underpinning of conifer terpenoid-based and other defenses against pests and pathogens is complex and multifaceted. Preceding the TPS-mediated reactions that produce the terpenoids in conifer resin are a number of other reactions contained in two pathways and compartmentalized on a subcellular level. Terpenoids are formed by the action of multiple TPS enzymes, many of which also produce multiple products. TPS genes appear to be differentially transcribed in different tissues following insect or other damage or application of MeJA. Following production of terpenoids via the action of TPS enzymes, the terpenoid products may be modified by the action of other enzymes such as cytochrome P450-dependent monooxygenases. The enzymes involved in these transformations and their regulation are not well understood in conifers. The interplay of the above factors that are influenced by the biotic

and abiotic environment of conifers results in complex blends of terpenoids in the oleoresin defenses and volatile emissions. It is possible that conifers defend themselves differently against different antagonists, and that insects and pathogens, or different species of each, may induce different blends of terpenoids that are, at least somewhat, optimized for defense against the specific threat. For example, foliage-feeding budworms, ovipositing sawflies, or stem-boring weevils, and bark beetles appear to induce different terpenoid responses in conifers. Additional work is needed to better understand the different responses and their specific fine-tuning.

Herbivore-, pathogen- or MeJA-induced biosynthesis of resin terpenoids is often accompanied by anatomical changes that allow the tree to deliver the terpenoid-laced resin to the site of the damage. Similarly phenolic defenses appear to be associated with specialized, inducible cell types in conifers. These anatomical and histological changes, mainly observed in the developing xylem of stems for terpenoids and in the bark for phenolics, will also have complex genetic foundations that are yet to be unraveled.

Natural selection should act on conifers to strengthen their ability to distinguish between antagonists, mount a rapid defense, include the terpenoids in their defensive secretions that will best counter the threat, and deliver their payload to the most appropriate location. Research has just begun to scratch the surface of the molecular genetic and biochemical foundations of these aspects of conifer defense systems. The recently developed genomics tools for conifers will unravel new aspects of defense against herbivores and pathogens in long-lived trees. Sustainability of conifer forests is facing new challenges under scenarios of climate-change-related increases in biotic and abiotic stress. The questions that remain outnumber, by far, the questions that have been answered to date. Research in this field will prove to be useful not only in a basic sense, but also in an applied manner. A more complete understanding of conifer natural defenses at the molecular and genomic levels will provide new strategies to complement existing and proven approaches for sustainable forestry for the future.

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REVIEW ARTICLE

GENE-FOR-GENE DISEASE RESISTANCE: BRIDGING INSECT PEST AND PATHOGEN DEFENSE

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Abstract—Active plant defense, also known as gene-for-gene resistance, is triggered when a plant resistance (*R*) gene recognizes the intrusion of a specific insect pest or pathogen. Activation of plant defense includes an array of physiological and transcriptional reprogramming. During the past decade, a large number of plant *R* genes that confer resistance to diverse group of pathogens have been cloned from a number of plant species. Based on predicted protein structures, these genes are classified into a small number of groups, indicating that structurally related *R* genes recognize phylogenetically distinct pathogens. An extreme example is the tomato *Mi-1* gene, which confers resistance to potato aphid (*Macrosiphum euphorbiae*), whitefly (*Bemisia tabaci*), and root-knot nematodes (*Meloidogyne* spp.). While *Mi-1* remains the only cloned insect *R* gene, there is evidence that gene-for-gene type of plant defense against piercing-sucking insects exists in a number of plant species.

Key Words—resistance genes, piercing-sucking insects, active plant defense, resistance response, *Mi-1*.

Plants are exposed to a large number of pests and pathogens. However, only a small proportion of these attacks and invasions are successful and result in disease. This is because plants have evolved to defend themselves from invading pests and pathogens (reviewed in Walling, 2000; Dangl and Jones, 2001). The first line of defense is passive and includes physical barriers like waxy or thick cuticles and the presence of specialized trichomes that inhibit insects or pathogens from settling, penetrating plant surfaces, and successfully colonizing plants. In addition to these

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physical barriers, there are two overlapping yet different forms of active plant defense. The first is known as the basal plant defense that restricts the invasion of a virulent pathogen or insect. The second involves specific recognition of the invading pest or pathogen by plant resistance (*R*) genes. Upon recognition of the attacking organism, plant defenses are initiated that serve to localize the invasion of the pathogen or deter feeding of the insect. This review focuses on *R* genes and the active form of plant defense against piercing-sucking insects, and highlights how these defense responses relate to those against plant–pathogens.

The genetic basis of plant resistance was first elucidated by Flor in the early 1940s (Flor, 1955). Studying the flax pathogen, *Melampsora lini*, Flor demonstrated that resistance to this fungus is due to the simultaneous presence of a *R* gene in the host and a matching avirulence (*Avr*) gene in the fungus. The absence of either the *R* gene or the *Avr* gene results in disease. This observation led to the theory of gene-for-gene complementarity between host and pathogen (Keen, 1990). The specific recognition of the *Avr* gene product facilitated by the plant resistance gene product triggers a signal transduction cascade that activates plant defenses. The gene-for-gene system is undoubtedly an oversimplification of the phenomenon; however, it has been a useful starting point for predicting plant–pathogen interactions (Dangl and Jones, 2001; Hammond-Kosack and Parker, 2003; Innes, 2004).

Insect R Genes

Resistance to insects has been identified in various plant species (Panda and Khush, 1995; Quisenberry and Clement, 2002). A number of single dominant *R* genes have been mapped, and molecular markers linked to these loci have been identified (Venter and Botha, 2000; reviewed in Yencho et al., 2000; Klingler et al., 2001; Liu et al., 2001; Jena et al., 2002; Liu et al., 2002a; Tan et al., 2004). The majority of the mapped genes are in staple crops like wheat and rice. The largest number of these mapped genes confer resistance to Hessian fly, *Mayetiola destructor*, which in addition to the Russian wheat aphid, *Diuraphis noxia*, is considered one of the most serious insect pests of wheat (Webster et al., 2000). In addition to these mapped genes, several single dominant aphid *R* genes have been identified that confer resistance to a single species of aphid. An example is the *Nr* (resistance to *Nasanova ribisnigri*) gene in lettuce conferring resistance to *N. ribisnigri* (Reinink and Dieleman, 1989).

During the last decade, a large number of *R* genes have been cloned from a number of plant species (reviewed in van't Slot and Knogge, 2002; Hammond-Kosack and Parker, 2003). Although these genes confer resistance to diverse groups of organisms, such as bacteria, virus, fungi, oomycetes, nematodes, and insects, their products share striking structural similarities. These structural similarities are also shared among *R* gene products from monocots and dicots, indicating that

recognition and activation of plant defense signal transduction has been maintained throughout evolution.

The most common structural motif shared by *R* genes is presence of the leucine-rich-repeat (LRR), which in other proteins facilitates protein–protein interactions (Kobe and Deisenhofer, 1994). In addition to the LRR domain, the largest class of *R* genes cloned to date contains a nucleotide binding site (NBS) of P-loop proteins (Traut, 1994) and a variable amino terminal region. Another less common class of R proteins, contains extracellular LRR with a transmembrane domain and either a kinase domain or a short intracellular carboxyl terminus (Hammond-Kosack and Parker, 2003; Nimchuk et al., 2003). A rare class of *R* genes encodes serine/threonine protein kinases (Martin et al., 1993; Swiderski and Innes, 2001). To date, the only cloned insect resistance gene is *Mi-1* (resistance to *Meloidogyne incognita*) from tomato. *Mi-1* belongs to the NBS–LRR class of *R* genes (Milligan et al., 1998; Rossi et al., 1998; Nombela et al., 2003).

The race to clone additional insect *R* genes has been accelerated by the advent of high throughput molecular tools, such as genome mapping, sequencing, and gene cloning. In addition, information gained from the sequences of currently cloned *R* genes is assisting in the search for candidate insect *R* genes (Brotman et al., 2002). With increasing numbers of molecular markers and high throughput methods, mapping *R* gene candidates and resistant phenotypes to each other is feasible. However, structural organization of *R* loci indicates that insect and disease *R* genes are clustered in plant genomes, making it more difficult to identify the gene family member conferring the resistance (McMullen and Simcox, 1996).

Clustering of members of multigene families in plant genomes is common for plant *R* genes. Examples exist where only a single gene in the cluster determines resistance. This is true for a number of loci including the *Mi* locus, the rust resistance locus *M*, the fungal resistance locus *Cf9 (resistance to Cladosprorium fulvum)*, the tobacco mosaic virus (TMV) resistance locus *N*, and the bacterial resistance locus *Pto (*resistance to *Pseudomonas syringae* pv. *tomato*), where an array of related genes is present in a cluster with only one member conferring disease resistance (Martin et al., 1993; Jones et al., 1994; Whitham et al., 1994; Anderson et al., 1997, Kaloshian et al., 1998). Therefore, cloning and transformation of candidate genes is required to identify their functional role.

The Structural Organization of the Mi-1 Locus

Resistance to root-knot nematodes mediated by the *Mi-1* gene was identified in a wild relative of tomato *Lycopersicon peruvianum*. *Mi-1* was introgressed from *L. peruvianum* into cultivated tomato, *Lycopersicon esculentum*, by using embryo rescue of an interspecific cross between these two *Lycopersicon* species (Smith, 1944). Progeny of a single F1 plant is the sole source of nematode resistance in currently available fresh-market and processing tomato cultivars (Medina-Filho

and Stevens, 1980). The *Mi-1* locus was localized to the short arm of tomato chromosome 6.

In addition to nematode resistance, resistance to potato aphid, *Macrosiphum euphorbiae,* was also identified within a 650-kb region of the short arm of chromosome 6 (Kaloshian et al., 1995). In this same region, two clusters of NBS–LRR type of *R* genes were identified (Vos et al., 1998), and the resistance to aphids and nematodes was localized to one of these clusters (Kaloshian et al., 1998). Within this cluster, two transcribed NBS–LRR type of *R* genes, *Mi-1.1* and *Mi-1.2*, with over 91% amino acid sequence identity and an apparent pseudogene, were identified. Even though the two genes were highly similar, only *Mi-1.2* (referred to in this article as *Mi-1*) conferred resistance to both root-knot nematodes and potato aphids (Milligan et al., 1998; Rossi et al., 1998). Later, it was shown that *Mi-1* also conferred resistance to both Q- and B- biotypes of *Bemisia tabaci* (Nombela et al., 2003). The function of *Mi-1.1* has not yet been identified.

The Mi-1 Surveillance System

The tomato gene *Mi-1* encodes a cytoplasmic protein of 1257 amino acids with putative coiled coil (CC) NBS–LRR domains (Milligan et al., 1998). Based on animal models, CC domains are presumed to be regions of protein–protein interacting domains and, therefore, may interact with partners involved in resistance signaling (Dubin et al., 2004). Recently, it was shown that the NBS domain has the ability to specifically bind and hydrolyze ATP (Tameling et al., 2002). ATP hydrolysis might provide the energy needed for a possible conformational change of NBS–LRR proteins, required to initiate signaling. ATP binding and hydrolysis have been shown to be necessary for signaling by proteins with NBS domains that regulate cell death in animal systems (van der Biezen and Jones, 1998).

Using genetics and functional studies, the LRR region of R proteins has been shown to determine recognition specificity (Botella et al., 1998; Meyers et al., 1998; Ellis et al., 1999; Bittner-Eddy et al., 2000; Dodds et al., 2001). This region is under selection for divergence, and it is the most variable region in closely related R proteins (Meyers et al., 1998). However, other protein domains in the LRR-containing *R* genes also have been implicated in determining recognition specificity (Luck et al., 2000). More recently, LRR and NBS regions have been implicated in intra- and intermolecular signaling and interaction with plant signal transduction components (Moffett et al., 2002; Liu et al., 2004). Mutations in NBS and LRR domains have resulted in either nonfunctional alleles or *R* gene products that are constitutively active in the absence of pathogen effector molecules (Bendahmane et al., 2002; Shirano et al., 2002). These data indicate that these *R* genes are under negative regulation.

Similarly, *Mi-1* is negatively regulated in the absence of nematodes or insects. Several chimeric constructs were made between the functional *Mi-1.2* allele

and the nonfunctional *Mi-1.1* allele (Hwang et al., 2000). Constructs were transformed into *Agrobacterium rhizogenes* and used in both transformation assays, which result in hairy root formation, and in transient expression in leaves. One of these constructs is Mi-DS4, produced by introducing the *Mi-1.2* LRR encoding region into *Mi-1.1*. Infiltration of *Nicotiana benthamina* leaves with *A. rhizogenes* containing Mi-DS4 results in cell death due to constitutive activation of the *Mi-1*-mediated cell death pathway (Hwang et al., 2000). Similarly,Mi-DS4 when used in *A. rhizogenes*-mediated transformation of cotyledons, failed to produce transformed roots due to the lethal phenotype. Using Mi-DS4 and other chimeric constructs between *Mi-1.2* and *Mi-1.1*, it was shown that intramolecular interaction in *Mi-1* regulates cell death, where the LRR region is involved in signaling cell death and the amino terminus region, which includes the CC and possibly the NBS domains, negatively regulating this signal (Hwang et al., 2000; Hwang and Williamson, 2003). In addition, these studies narrowed down the region of *Mi-1* important for recognition of the nematode effector protein or a plant protein that determines specificity to a segment of three amino acid residues, 984–986, which are located in the LRR domain of *Mi-1*.

Additional genetic evidence indicates that another gene, *Rme1* (required for resistance to *Meloidogyne*), is required for the *Mi-1*-mediated resistance to aphids, whiteflies, and nematodes (Martinez de Ilarduya et al., 2001, 2004). The *rme1* mutant was isolated in a genetic screen of mutagenised *Mi-1* tomato populations. The *rme1* mutant had a functional *Mi-1* gene, but was compromised in resistance to root-knot nematodes (Martinez de Ilarduya et al., 2001). Later, this mutant was also shown to be compromised in resistance to potato aphids and whiteflies (Martinez de Ilarduya et al., 2001, 2004).

To determine if *Rme1* acted upstream or downstream of *Mi-1*, the phenotype of plants expressing the mutant *rme1* allele and the chimeric Mi-DS4 were evaluated (Martinez de Ilarduya et al., 2004). Mi-DS4 was introduced into *rme1* cotyledons via *A. rhizogenes*-mediated transformation. These plants failed to produce transformed roots, indicating that while resistance was compromised in the *rme1* mutant, the cell death pathway remained active. Therefore, it is possible that *Rme1* interacts with *Mi-1* at amino acid residues 984–986 of the LRR.

The "Guard Theory" and R Protein Complexes

The gene-for-gene hypothesis predicts direct interaction between R proteins and Avr effector proteins (Keen, 1990). Direct interaction of NBS–LRR type of R proteins with corresponding effector proteins remains the exception rather then the rule (Jia et al., 2000; Deslandes et al., 2003). According to the emerging "guard theory," NBS–LRR plant R proteins monitor the interaction of another plant protein with the pathogen or pest Avr determinant (Dangl and Jones, 2001). The pathogen or pest Avr determinant targets this host protein, which might be

part of general plant defense, to suppress defense responses. It is speculated that Avr–host protein interaction results in conformational change in the target host protein, which allows binding by the plant R protein. The binding of the R protein to this target protein or complex in turn triggers the incompatible response. In the absence of the R protein, the host defenses are suppressed by the pathogen or pest virulence determinant, and disease or compatible response follows.

Several examples supporting the guard theory exist. A notable one is the *Arabidopsis* NBS–LRR gene *RPS5* (Resistance to *Pseudomonas syringae),* which confers resistance to *P. syringae* expressing the *Avr* gene A*vrPphB*. The resistance mediated by *RPS5* requires a protein kinase *PBS1* (*AvrPphB* susceptible) (Warren et al., 1999; Swiderski and Innes, 2001). Recently, it has been shown that AvrPphB, a cysteine protease, binds PBS1 and cleaves it. This cleavage triggers *RPS5*-mediated resistance, indicating that RPS5 might sense the integrity of PBS1 (Shao et al., 2002, 2003).

The guard hypothesis proposes indirect interaction between NBS–LRR type of R proteins and their corresponding Avr determinants. Both *Arabidopsis* RPM1 (Resistance to *P. syringae* pv. *maculicola*) and RPS2 (Resistance to *P. syringae* expressing *AvrRpt2*) R proteins interact with their corresponding Avr proteins through RIN4 (RPM1 interacting protein), and RIN4 is the target of three different bacterial effector proteins AvrRpt2, Avr RPM1, and AvrB (Mackey et al., 2002, 2003; Axtell and Staskawicz, 2003).

Based on these and other models, it is intriguing to postulate a role for *Rme1* in resistance to both aphids and nematodes. It is possible that *Rme1* is the target of the nematode and insect effector molecules (Figure 1). These animal Avr products may induce a conformational change in *Rme1*. *Mi-1* could be monitoring these changes in *Rme1* brought about by the interaction with nematode, aphid, or whitefly Avr determinants and trigger rapid activation of defenses (Figure 1). In the absence of *Mi-1* or *Rme1*, the feeding by insects or by nematodes is not detected and attack continues.

The interaction between *Mi-1* and *Rme1* is likely to be more complex. Recent data suggest that R proteins are associated in multimeric complexes with a number of plant proteins that are involved in both signaling and regulation (reviewed in Shirasu and Schulze-Lefert, 2003; Schulze-Lefert, 2004). One of these plant proteins present in a number of R protein complexes is the molecular chaperone Hsp90 (heat shock protein 90). Chaperonins, like Hsp90, are known to play a role in assembly and stability of multisubunit complexes. Requirement for Hsp90 has been shown for the function of NBS–LRR type of *R* genes *RPS2* and *RPM1* of *Arabidopsis*, the tobacco *N*, and the potato virus X (PVX) resistance gene, *Rx* (resistance to PVX) (Hubert et al., 2003; Lu et al., 2003; Takahashi et al., 2003; Liu et al., 2004). Similarly, by using virus-induced gene silencing of *Hsp90* transcripts, a role for *Hsp90* in *Mi-1*-mediated

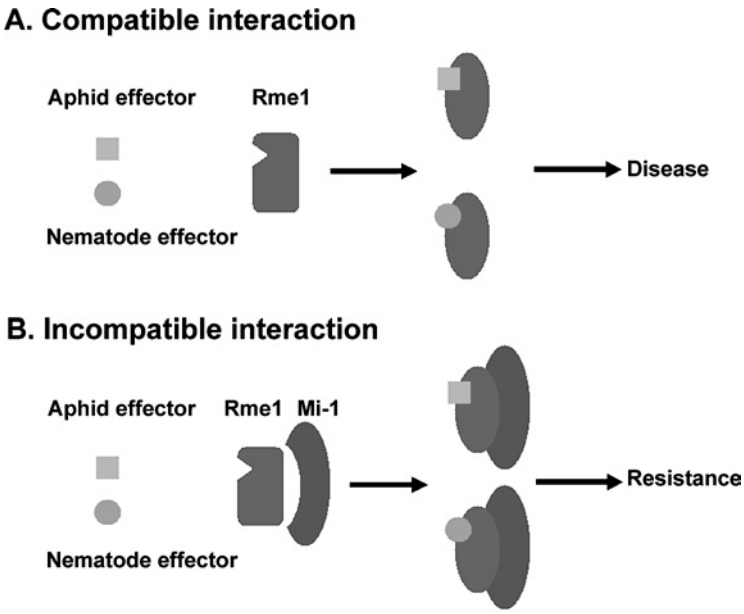

FIG. 1. A schematic model diagram, depicting early interactions in the *Mi-1* signaling pathway. Based on the guard theory, Rme1 is depicted as the target for the insect and nematode effector molecules. Interaction with both aphid and nematode effector molecules results in a conformational change of Rme1 protein. (A) In the absence of *Mi-1*, insect and nematode effector molecules interact with Rme1 which initiates a compatible interaction resulting in disease. (B) In the presence of *Mi-1*, a conformational change in Rme1 due to these interactions is detected, and defense responses or an incompatible interaction is triggered resulting in resistance.

resistance to both nematodes and aphids has been identified (Kaloshian, unpublished results).

Protein complexes containing R protein and Hsp90 could also contain other plant proteins, like Sgt1 (suppressor of G_2 allele of SKP1) and Rar1 (required for *Mla*12 resistance). These proteins might assist in proper assembly of the R complex into a stable signaling competent complex (Schulze-Lefert, 2004). The requirement for *Sgt1* and *Rar1* has been implicated in a number of NBS–LRR *R* gene-mediated resistance in plants as diverse as barley, tobacco, and *Arabidopsis* (reviewed in Shirasu and Schulze-Lefert, 2003). The requirement for *Sgt1* and *Rar1* implicates a role for the ubiquitination pathway, which targets proteins for degradation, in regulating *R* gene signaling (Azevedo et al., 2002; Liu et al., 2002b).

Avirulence Effectors

Complex communications occur between host and the invading pest or pathogen. To successfully infect and colonize host plants, microbial plant pathogens deliver effector molecules into their host cells. In order to reach their feeding sites and deliver these effector molecules, both piercing-sucking insects and nematodes penetrate their hosts with a combination of mechanical penetration and hydrolytic enzymes (reviewed in Miles, 1999; Rosso et al., 1999; Wang et al., 1999; Popeijus et al., 2000; reviewed in Davis et al., 2004). They use their stylets as a mechanical tool, as well as for delivery of various types of molecules to plant tissues and for ingestion of nutrients from phloem or feeding sites.

During the penetration and feeding processes, two types of saliva, gelling and watery saliva, are secreted by aphids and whiteflies. Gelling saliva, also known as the sheath saliva, is secreted along the path of the stylet and contains mainly proteins, phospholipids, and conjugated carbohydrates (reviewed in Miles, 1999). In addition to these components, enzymes like phenoloxidases and peroxidases, and 1,4-glucosidases has also been reported to occur in the sheath saliva (Urbanska et al., 1998; Miles, 1999; Cherqui and Tjallingii, 2000). Watery saliva contains variable and complex arrays of enzymes including phenoloxidases, peroxidase, pectinase, amylases, alkaline and acidic phosphatases, and lipases (Miles, 1999; Cherqui and Tjallingii, 2000). It is likely that piercing-sucking insect Avr determinants are present and delivered by the watery saliva into plant cells, or salivary components aid in generating oligosaccharides and cell wall fragments or other defense signals *in planta* that activate plant defenses.

There is ongoing research to uncover Avr effectors of insects and nematodes (Semblat et al., 2001; Rider et al., 2002; Williamson and Gleason, 2003). To date, no insect or nematode Avr effector proteins have been identified conclusively. In plant pathogen systems, both proteinaceous and nonproteinaceous effectors have been identified (reviewed in van't Slot and Knogge, 2002). The functional role of only a subset of these Avr molecules has been identified. In viral pathogens coat proteins, replicase proteins and movement proteins have been shown to act as Avr effectors (reviewed in van't Slot and Knogge, 2002). Recently, in bacterial and fungal pathogens, Avr proteins possessing protease activity have been identified (Jia et al., 2000; Shao et al., 2002, 2003). Because of the diversity of known effector molecules from bacteria, fungi, and viruses, it is difficult to speculate the nature of these molecules in insects and nematodes. Most likely, the nature of Mi-1 effectors from nematode, aphid and whitefly are different. However, conserved signature motifs in the effector molecules from root-knot nematodes, potato aphids, and whiteflies may interact directly with *Mi-1* or with *Rme1*, or in association with other plant components, to trigger the resistance response. It is also possible that *Mi-1* or *Rme1* recognizes more than one effector molecule as is the case with *RIN4* and the tomato *R* gene *Pto*, conferring resistance to *P. syringae* pv. *tomato* with two different *Avr* genes (Kim et al., 2002).

Since the plant surveillance system detects Avr molecules, why would a pathogen or pest maintain these? It is becoming increasing apparent that some of the effector molecules are maintained in populations of pathogens because they also function as virulence factors (reviewed in Kjemtrup et al., 2000). Virulence factors are required for the full success of a pest or pathogen on a susceptible host. Therefore, in both resistant and susceptible plants, a plant protein is targeted by the pathogen or pest virulence effector molecules. In plants lacking a *R* gene, disease ensues or pest attack continues. While in resistant plants, the Avr–host protein interaction is detected by the R protein, and defense responses are rapidly activated. This is the basis of the "guard theory" described above (Dangl and Jones, 2001) (Figure 1).

A number of insects of the order Hemiptera have endosymbionts which play an essential role in the life of these insects (reviewed in Baumann et al., 1995). The potential role of these microorganisms in plant–insect interactions has not yet been uncovered but cannot be ignored. It is plausible that avirulent effectors from this group of insects could be of bacterial origin.

R Gene-mediated Specificity and Resistance Mechanisms

Plants are in a continuous race with their pests and pathogens. As plant *R* genes evolve to acquire new recognition specificities, pathogens and pests find new ways to circumvent this new recognition machinery. Consequently, any particular single source of resistance, when used in monoculture, is only effective in the field for a short period of time. Most gene-for-gene type insect resistance deployed in crops behave in the same way. In addition, they confer resistance to a single species and limited biotypes of the insect (Hatchett and Gallun, 1970; Ratcliffe and Hatchett, 1997). This is also true for aphid resistance mediated by *Mi-1* where resistance is limited to only *M. euphorbiae* and certain biotypes of this aphid (Goggin et al., 2001).

Recognition of the Avr effectors by the host R protein initiates a defense response that is often, but not always, characterized by hypersensitive response (HR). The HR is a programmed cell death that is initiated at the site of the infection or feeding (Morel and Dangl, 1997). HR is associated with the resistance response mediated by the *Mi-1* gene, in roots, against root-knot nematodes (Dropkin, 1969). Localized necrotic spots are seen near the head of the infective-stage of the nematode, soon after it initiates feeding near the vasculature, presumably limiting access to nutrients. However, in the resistance response to potato aphid, no HR is seen in tomato leaflets infested by the aphid (Martinez de Ilarduya et al., 2003). Nevertheless, induction of HR is not absolutely necessary for activation of plant defense to pathogens. Absence of HR has been reported in plant–pathogen interactions where induction of defense responses was not correlated with initiation of HR (Jakobek and Lindgren, 1993; Cameron et al., 1994; Jia and Martin, 1999).

It is not clear whether HR is a common resistance response to piercing-sucking insects. For example, both presence and absence of HR have been reported in incompatible interactions between wheat and Hessian fly (Hatchett et al., 1993; Grover, 1995; C. Williams, personal communication). Irrespective of presence or absence of HR, a common mechanism of *R* gene-mediated resistance to piercing-sucking insects seems to be limited phloem-feeding (van Helden et al., 1993; Klingler et al., 1998; Kaloshian et al., 2000). Interestingly, the mechanisms of resistance mediated by *Mi-1* to potato aphids and whiteflies appear to be different. Electrical penetration graph studies show that on *Mi-1* plants, aphids do not ingest phloem and die from starvation (Kaloshian et al., 2000). In contrast, whiteflies are able to feed on resistant tomato phloem sap, but have difficulty reaching the phloem element in resistant plants (Jiang et al., 2001). This indicates that *Mi-1*-mediated defense responses are perceived differently by these two insects.

R GENE-MEDIATED PLANT DEFENSE TO INSECTS

There is increasing evidence that *R* gene-mediated resistance is a hyperactivity of basal plant defense. In other words, *R* gene-mediated resistance is a more efficient defense response than the basal response. The existence of basal plant defense responses was genetically dissected during the last decade (reviewed in Glazebrook, 1999, 2001). Using mutational analysis, it was discovered that it is possible to obtain plants that are more susceptible than existing susceptible genotypes. One of the initial screens was performed with *P. syringae* pv. *maculicola*. A high dose of the moderately pathogenic bacterium *P. syringae* pv. *maculicola* results in water-soaked lesions on *Arabidopsis* leaves. In contrast, a low dose results in limited chlorotic spots. Using a low dose of this bacterium and a mutagenized *Arabidopsis* population, several mutants were identified with enhanced disease susceptibility. Among those were the *eds* (enhanced disease susceptibility) mutants, *pad* (phytoalexin deficient) mutants, and the *npr1* (nonexpresser of pathogenesis related gene) mutant (Cao et al., 1994; Glazebrook and Ausubel, 1994; Glazebrook et al., 1996).

Microarray analysis of gene expression profiling comparing *R* gene-mediated incompatible responses to compatible responses, indicates that defense genes are activated faster and to higher levels in incompatible compared to compatible interactions (Tao et al., 2003). Indeed the hallmark of *R* gene-mediated resistance historically has been faster and higher level accumulation of pathogenesis-related (*PR*) gene transcripts in incompatible interactions compared to compatible interactions (Somssich et al., 1989). This pattern is also seen for *PR-1* gene expression in the *Mi-1*-mediated incompatible interaction compared to the compatible interaction after potato aphid infestation (Martinez de Ilarduya et al., 2003). Similarly, increases in chitinases and β-1,3-glucanases activity have been reported in

incompatible interactions of monocots infestation with aphids (van der Westhuizen et al., 1998; Forslund et al., 2000). Recent gene expression profiling by array analysis indicates that resistance in wheat to Russian wheat aphid activates the oxidative stress pathway similar to pathogen-induced *R*-mediated resistance responses (E. Boyko, personal communication).

Plant defense responses to pathogens include pathways dependent on salicylate (SA), jasmonate (JA), and ethylene (ET) signaling molecules. These pathways are not independent of each other, rather a complex network of communication between these pathways results in modulation of plant defense. Both synergistic and antagonistic interactions between SA, JA, and ET signaling pathways have been reported (reviewed in Felton and Korth, 2000; Kunkel and Brooks, 2002). These interactions and consequent modulation of plant defenses are different in different plant species and plant–pathogen or plant–pest interactions. In nature, plants are often simultaneously attacked by a number of different organisms. The cross talk between SA, JA, and ET signaling pathways allows the plant to choose the optimum plant defense, depending on the nature and combination of the attackers.

Both SA- and JA-dependent pathways have been implicated in basal defense responses to phloem- and sap-feeding insects (Fidantsef et al., 1999; Walling, 2000; Moran and Thompson, 2001; Moran et al., 2002; Martinez de Ilarduya et al., 2003; Zhu-Salzman et al., 2004). Using *Arabidopsis* genetic mutants *eds5*, *eds9*, and *npr-1*, defective in SA signaling, resulted in no change in aphid reproduction from that on wild-type plants (Moran and Thompson, 2001). In contrast, higher reproduction of aphids was observed on a *coi-1* (coronatine insensitive) mutant (defective in JA response) and lower reproduction on *cev-1* (constitutive expression of vegetative storage protein) mutant (constitutive JA and ET response) compared to wild-type plants, indicating a role for jasmonate pathway in aphid defense (Feys et al., 1994; Ellis and Turner, 2001; Ellis et al., 2002). Taken together, these results indicate that both JA and SA pathways might be involved in aphid resistance in *Arabidopsis*.

Accumulation of *PR-1* transcripts after aphid feeding on *Mi-1*-containing tomato plants, also indicates a role of SA in resistance to aphids (Martinez de Ilarduya et al., 2003). Stronger evidence for the role of SA in *Mi-1*-mediated resistance was recently demonstrated. *NahG* transgene, which encodes salicylate hydroxylase and degrades SA into catchecol, was introduced into *Mi-1*-containing tomato (Branch et al., 2004; Kaloshian, unpublished results). Expression of *NahG* reduced the levels of SA in both root and leaf tissues and significantly reduced the *Mi-1*-mediated resistance to root-knot nematodes and potato aphids (Branch et al., 2004; Kaloshian, unpublished results). The loss of *Mi-1*-mediated resistance was rescued using a SA functional analogue, benzothiadiazole, indicating that SA and not yet undetermined defense pathways, which are also affected by *NahG* expression, are involved in *Mi-1*-mediated resistance (Heck et al., 2003; Branch et al., 2004; Kaloshian, unpublished results).

JA and ET have also been shown to regulate basal defense to whiteflies in tomato and to silverleaf whiteflies in squash, indicating that, generalization of involvement of a specific defense pathway cannot be made based solely on insect guild (van de Ven et al., 2000; Walling, 2000).

Evaluation of plant defense by SA signaling pathway is being reconsidered because of a significant recent finding (Wildermuth et al., 2001). The known route for SA synthesis was by converting chorismate, synthesized by the shikimate pathway, through the phenylpropanoid pathway into SA. However, recent reports indicate a minor role of the phenylpropanoid pathway in SA biosynthesis for plant defense (Metraux, 2002). Wildermuth et al. (2001), suggested that for plant defense, SA is synthesized in the chloroplast from chorismate by isochorismate synthase 1 into isochorismate, which in turn is converted into SA. It remains to be seen whether this novel branch of SA synthesis, which is known to operate in prokaryotes, is also present in other plant species (Serino et al., 1995).

In addition to these known pathways, plant defenses independent of JA, SA, or ET have been also identified (Glazebrook et al., 2003). In sorghum, novel defense pathway(s) independent of JA and SA have also been implicated in aphid defense (Zhu-Salzman et al., 2004). Similarly, messages of a novel whitefly defense gene, *SLW3* (silverleaf whitefly-induced 3), do not accumulate after defense or wound-induced signals, indicating the presence of yet unidentified insect defense pathway(s) (van de Ven et al., 2000; Walling, 2000).

A number of transcription factors seem to be involved in regulation of defense responses (reviewed in Rushton and Somssich, 1998; Eulgem et al., 2000). Further evidence for involvement of these transcription factors in activation of plant defenses comes from the presence of putative binding sites in *PR* gene and other defense gene promoters (reviewed in Rushton and Somssich, 1998). Similarly, the plant-specific WRKY-type transcription factor has been implicated in control of *Hfr-1* (Hessian fly-response gene 1), which encodes a mannose-binding jacalin-like lectin (Williams et al., 2002; C. Williams, personal communication). *Hrf-1* is up-regulated in the incompatible response to Hessian fly mediated by *H*9 wheat resistance gene. Although it is not clear if *Hrf-1* encoded protein has lectin activity, this type of lectins may coat the midgut of the larvae, reducing absorption of nutrients (Foissac et al., 2000; Williams et al., 2002).

Future Directions

Development and accessibility of molecular tools during the past decade has resulted in the generation of a significant amount of information about plant–insect interactions. However, there is still much to be learned about these interactions. Cloning additional insect *R* genes should be a top priority and will allow an understanding of the spectrum of *R* gene motifs and domains in *R* genes that are specific for insect recognition. This will assist in exploiting the natural diversity of insect

R genes and facilitate the identification of resistance sources in wild germplasms. Incorporation of resistance sources by pyramiding *R* genes in cultivated species will assist in developing durable resistance. In addition, identifying nonbiotype specific *R* genes like the *Arabidopsis RPW8* (resistance to powdery mildew8) locus, which confers resistance to a wide range of powdery mildews, will be a very useful tool in the fight against insect pests (Xiao et al., 2001).

Gene expression profiling is generating a large amount of information about insect plant defense and identifying novel genes and pathways. It is clear that plants perceive and respond to different stimuli by modulating a number of defense pathways, and the interactions between these pathways vary among plant species. Therefore, it will be important to study plant–insect interactions in more than a single plant species. The next and important phase of research is to assess the functional roles of the upregulated genes, identified from expression profiling studies, and determine their contribution to plant defense. Recent advances in gene silencing that use virus-induced gene silencing or RNA interference techniques will allow these questions to be addressed (Ruiz et al., 1998; Chuang and Meyerowitz, 2000).

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CHARACTERIZATION OF A SALIVARY LYSOZYME IN LARVAL *Helicoverpa zea*

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Abstract—The cDNA sequence of a salivary lysozyme in *Helicoverpa zea* (Lepidoptera: Noctuidae) was determined. The full-length cDNA is 1,032 bp, and it encodes a protein of 142 amino acids. This lysozyme has 90% identity with *Heliothis virescens* lysozyme and 76% identity with *Manduca sexta* lysozyme. There is a signal peptide of 20 amino acids at the N-terminus. The mature protein is about 14.4 kDa without the signal peptide. The pI value is greater than 9.5 as determined by isoelectric focusing. From genomic DNA, two introns and three exons were within the open reading frame (ORF). Southern blot analysis indicated that it is a single-copy gene. A time-course study revealed that the *H. zea* lysozyme gene was differentially expressed in the labial glands during the development of fifth-instar larvae, with the peak level of lysozyme mRNA being detected on day 1. Dot blot analysis showed different levels of *H. zea* lysozyme expression when the caterpillars fed on different plants. Further, the *H. zea* lysozyme could be detected with antibodies raised against the *M. sexta* lysozyme, and it was one of the most abundant secreted proteins in saliva collected directly from the caterpillar's spinneret. The potential role of the lysozyme on host plants in mediating susceptibility to bacterial disease is discussed in the context of tritrophic interactions.

Key Words—Lysozyme, tritrophic interactions, saliva, plant signaling, *Helicoverpa zea*.

INTRODUCTION

One of the key functions of arthropod saliva is to facilitate feeding and digestion (Felton and Eichenseer, 1999). Solid food is solubilized by saliva before ingestion (Eliason, 1963; Miles, 1972). Many enzymes in the saliva may initiate food digestion prior to the food bolus reaching the alimentary canal (Miles, 1972; Ribeiro,

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1995). In some blood-feeding insects, salivary glands have an impact on the probing process and on modulation of cibarial pump frequency in live hosts (Ribeiro, 1995; Sant' Anna et al., 2001). Most blood-feeding arthropods have various salivary components that help to overcome their host defensive responses, including hemostasis, inflammation, and immunity (Ribeiro and Francischetti, 2003). In herbivores such as aphids, the salivary proteins can detoxify defensive phytochemicals (Peng and Miles, 1988; Miles and Peng, 1989). Saliva may facilitate pathogen transmission in blood-sucking arthropods and plant virus transmission in insect vectors (Sylvester, 1962; Miles, 1968; Ribeiro, 1989). Salivary glands may also be involved in water regulation, pheromone production, and antimicrobial defense (reviewed in Ribeiro, 1995).

Salivary glands are the largest exocrine glands in most arthropods (Ribeiro, 1995). In lepidopteran larvae, there are two pairs, the labial glands (also known as silk glands) and the mandibular glands (Saxena, 1972; Felton and Eichenseer, 1999). Labial glands are often comparatively large and long, almost running the length of the entire body. Watery secretions and silk from the labial glands are released from specialized structures called the spinnerets (Carter and Hargreaves, 1986; Felton and Eichenseer, 1999). Mandibular glands are frequently smaller, and the secretions are released from the pores on the mandibles (Felton and Eichenseer, 1999). Saliva refers to the secretions from the salivary glands, which should be distinguished from the regurgitant (frequently called oral secretions) originating mostly from the gut and released from the oral cavity.

In lepidopteran insects, most of the early studies on saliva focused on silk formation and digestive enzymes. Various carbohydrases (e.g., amylase, maltase, invertase, lactase, trehalase, saccharase), and proteases (e.g., pepsin, aminotripeptidase, leucine aminopeptidase, dipeptidase, prolinase), as well as lipases, have been found in the Lepidoptera (Mathur, 1966; Chattoraj and Mall, 1969; Saxena, 1972; Verma and Balyan, 1972; Shimada and Hayashiya, 1975; Agarwal, 1976; Verma et al., 1977; Raghavan et al., 1978). In latter work, salivary components were also shown to be related to the larval molting process (Epstein and Lockshin, 1981; Gelman et al., 1991; Zheng et al., 2003). Recently, Eichenseer et al. (1999) found glucose oxidase in the labial glands and saliva of the bollworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae). Glucose oxidase is responsible for decreasing nicotine induction in tobacco fed on by *H. zea* (Musser et al., 2002). This enzyme may be one of the candidate factors that suppress host defensive responses by inhibiting the jasmonic acid signaling pathway (Eichenseer et al., 1999; Felton and Eichenseer, 1999; Musser et al., 2002).

The role of saliva in immunity is frequently overlooked. One prophenoloxidase-activating enzyme involved in antimicrobial activity was detected in the salivary glands of *Bombyx mori* (Satoh et al., 1999). Lysozyme has been characterized in the salivary glands of *Drosophila melanogaster* (Kylsten et al., 1992), the mosquitoes *Anopheles darlingi* (Moreira-Ferro et al., 1998,

1999) and *Aedes aegypti* (Valenzuela et al., 2002), and the termites *Hodotermopsis japonica* (Miura et al., 1999) and *Reticulitermes speratus* (Fujita et al., 2001, 2002). Lysozyme has antibacterial activity; it breaks down the bacterial cell wall peptidoglycan and lyses the cell. As far as we are aware, lysozyme has rarely been reported in the salivary glands of any species in Lepidoptera. In this paper, we report the characterization of a salivary lysozyme from *H. zea*.

METHODS AND MATERIALS

Insects. H. zea eggs were purchased from North Carolina State University Insectary. Larvae were reared on artificial diet (Jacob and Chippendale, 1971) at 27°C with a photoperiod of 14:10 hr L:D.

Collecting Saliva and Regurgitant. Last-instar *H. zea* larvae were fed for 2 days and then starved overnight and anesthetized on ice. Larvae were then removed from the ice, held in position with a clip, and saliva was collected from the spinneret with a microcapillary tube. Each tube was filled with *ca.* 1 μl of 0.1 M potassium phosphate buffer (pH 6.24) to avoid drying of saliva. Regurgitant was collected from the buccal cavity of last-instar larvae with a pipettor.

RNA and DNA Extraction. Total RNA was extracted from 30 pairs of labial glands from last-instar *H. zea* using TRIzol Reagent (Invitrogen, Calsbad, CA). To avoid hemolymph contamination, dissected labial glands were washed with ice-cold diethyl pyrocarbonate (DEPC)-treated phosphate buffered saline (PBS) five times. *H. zea* genomic DNA was extracted from ready-to-molt, last-instar larvae using a standard protocol (Ausubel et al., 1994).

cDNA Cloning and Sequencing. Approximately 1 μg of total RNA from 2-day-old larvae was used to perform first-strand cDNA synthesis using the SMART™(Switching Mechanism At 5′ end of RNA Transcript) RACE (rapid amplification of cDNA ends) cDNA Amplification Kit (Clontech, Palo Alto, CA). To amplify cDNA ends of lysozyme, degenerate primer Lyz-Rd was used (Table 1). 3′-RACE PCR was carried out using primers Lyz-Rd and Universal Primer A Mix (UPM) containing UPM long and UPM short. The amplified product was cloned into Topo-TA cloning vector (Invitrogen). The recombinant plasmid was purified, and the sequencing reaction, was performed using the BigDye sequencing kit (Applied Biosystems, Foster City, CA). All sequences were determined with an ABI version 3.0 automatic DNA sequencer (The Pennsylvania State University Nucleotide Sequencing Facility). Based on the sequencing results, gene-specific primers Lyz-F1 and Lyz-R1 were designed, and 5′-RACE and 3′-RACE polymerase chain reactions (PCR) were performed using primer pairs Lyz-F1 and UPM, and Lyz-R1 and UPM, respectively. Lyz-F1 and Lyz-R1 were then used as primers in the BigDye sequencing reaction.

TABLE 1. OLIGONUCLEOTIDES

Primer	Sequences (5′–3′)
Lyz-Rd	CCTTCAAYGGMTGGTACATG
UMP long	CTAATACGACTCACTATAGGGCAAGCA GTGGTATCAACGCAGAGT
UMP short	CTAATACGAGTGAGTATAGGGC
Lyz-F1	GGCGCAAGTCGACGCTTTGG
Lyz-R1	CAGTACTCCTGGGAAGGACTG
Lyz-F2	AATGTATAATACATAATCGGT
Lyz-R2	TGTGTGAACTAGGAAACAT
Lyz-F3	TGCGGCCGCAAGCTTTAATATCCGTGAAAATATGTAGGT
Lyz-F4	TCGATGTTGGATGTCAACT
Lyz-F5	CCCACTTTGTCTGTCTTCCT
Lyz-F6	TCGCAAGCGCAACCACGAACAA
Lyz-R3	GTCGTGTTGCATTGCGATGCGA
Lyz-R4	GAGGCGTGGTACGGATGGAA
Lyz-R5	AGTCTCAGATCTGGCAGA

In order to determine whether there were introns inside the lysozyme open reading frame (ORF) region, one set of gene-specific primers, Lyz-F2 and Lyz-R2, were designed for PCR on genomic DNA. Thirty-five cycles of amplification were done with *Taq* polymerase (Promega, Madison, WI) under the following conditions: 94°C for 30 sec, 50°C for 30 sec, and 72°C for 2 min. The PCR product was cloned and sequenced with primers Lyz-F3, Lyz-F4, Lyz-F5, Lyz-F6, Lyz-R3, Lyz-R4, and Lyz-R5.

Southern and Northern Hybridizations. For genomic Southern, 10 μg of genomic DNA were digested by *Xho*I and resolved on a 0.7% agarose gel. After denaturation and neutralization, the DNA was transferred to a nylon membrane. For northern analysis, 10 μg of total RNA were run on a 1% agarose gel and transferred to a nylon membrane. To study differential gene expression, 10 μg of total RNA of labial glands from *H. zea* larvae of different ages (0, 1, 2, and 3 days of the fifth instar), or feeding on different diets (artificial diet, cotton, tobacco, and tomato) were loaded on a nylon membrane in a dot blot format. A 458 bp PCR product of *H. zea* lysozyme cDNA was labeled by [α-^{32}P] dATP using the Prime-A-Gene System (Promega, Madison, WI). High stringency hybridization and washes were performed by using standard protocols (Sambrook et al., 1989).

SDS-PAGE and Western Blot Analysis. Labial glands from last-instar larvae of different ages (0, 1, 2, and 3 days) were dissected and homogenized in ice-cold 1x PBS (pH 8) buffer. After centrifuging at 5,000*g* for 5 min, the supernatant was transferred to a new tube. The extracted proteins from the labial glands, regurgitant, and saliva were separated by SDS-PAGE on a 15% gel (Laemmli, 1970). The saliva and regurgitant proteins were silver-stained (Blum et al., 1987), labial gland proteins were Coomassie blue-stained. Western blot was done by using a standard procedure (Sambrook et al., 1989) with a rabbit antiserum against the

Manduca sexta lysozyme (a gift from Dr M. Kanost, Kansas State University) at 1:2,000 as the primary antibody. Secondary antibodies were horse-radish peroxidase conjugated goat anti-rabbit IgG (1:2,500) (Sigma-Aldrich, St Louis, MO). The membrane was developed by using the DAB substrate (Roche, Indianapolis, IN). To increase sensitivity, membranes containing the salivary proteins were stained using the Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA).

Peptide Sequencing. Proteins from *H. zea* labial glands were separated by SDS-PAGE and blotted onto a PVDF (Bio-Rad, Hercules, CA) membrane. The membrane was stained by Coomassie blue for a few sec and washed with water. The target peptide band was cut out and sequenced on a Procise Sequencer (Applied Biosystems) (The Pennsylvania State University Peptide Sequencing Facility).

Isoelectric Focusing. Supernatants from two hundred pairs of labial glands were combined with 3 M urea to 17 ml, and 1 ml Bio-Lyte ampholytes (pH range 3–10, Bio-Rad) and 100 μl of 200 mM phenylmethylsulfonyl fluoride(PMSF) were added. The sample was loaded into the mini focusing chamber of a Rotofor system (Bio-Rad). Following the manufacturer's protocol, the polypeptides were separated into 20 fractions based on their isoelectric points. All fractions were concentrated with Nanosep 3 K centrifuge tubes (Pall, Noblesville, IN) and tested for lysozyme activity. The lysozyme substrate was the bacterium *Micrococcus lysodeikticus* (Sigma-Aldrich) (6 mg lyophilized cells dispersed in 15 ml potassium phosphate buffer). Each concentrated Rotofor fraction (10 μl) was loaded in duplicate into a 96-well microplate, and then the substrate (190 μl) was added into the well. The absorbance was read continuously for 15 min at 450 nm. Enzyme activity was expressed as one unit equals to absorbance change (ΔA450) of 0.001 per min at pH 6.24, 20°C. The specific activity was the enzyme activity divided by protein concentration that was measured by the Bradford Assay (Bradford, 1976) with bovine serum albumin (Sigma–Aldrich) as a standard.

Data Analysis. The *H. zea* lysozyme cDNA and protein sequences were aligned with other related lysozyme sequences by the software ClustalW (1.82) (http://www.ebi.ac.uk/ clustalw) (Thompson et al., 1997). Signal peptide was predicted by the program SignalP V1.1 (http://www.cbs.dtu.dk/services/SignalP/). A phylogenetic tree was constructed by using the neighbor-joining method of the MEGA 2.1 program (Kumar et al., 2001). Statistical analysis was carried out with MINITAB 13 (Minitab Inc., State College, PA).

RESULTS

Cloning and Sequencing of Lysozyme cDNA and Partial Genomic DNA. After reverse transcription of labial gland total RNA, we performed 3′-RACE using primer UPM and degenerate primer Lyz-Rd. A *ca*. 1 kb fragment was amplified, cloned, and sequenced. BLAST search showed that this sequence was highly

homologous to lysozyme sequences. Two other primers, Lyz-F1 and Lyz-R1, were designed based on this sequence and used to perform 5′- and 3′-RACE, respectively. Two overlapping fragments were obtained and sequenced. The composite full-length cDNA was 1,032 bp, and it contained an ORF encoding a putative lysozyme of 142 amino acids (Figure 1). There was a 48 bp untranslated region (UTR) at the 5′ end and a long 557 bp UTR at the 3′ end with a putative polyadenylation signal. The putative *H. zea* lysozyme protein contains a signal peptide of 20 amino acids as predicted by the program SignalP V1.1.

To determine whether the *H. zea* lysozyme gene is spliced, primers were designed corresponding to the cDNA sequence and used in PCR with *H. zea* genomic DNA as the template. Sequencing of the PCR products revealed two introns of 452 and 540 bp within the *H. zea* lysozyme ORF (Figure 1). Genomic Southern using labeled *H. zea* lysozyme cDNA as the probe detected a single band on the blot (Figure 2), suggesting that *H. zea* has a single-copy lysozyme gene.

Sequence Comparison. BLAST searches of the GenBank identified significant homology between the putative *H. zea* lysozyme with other insect lysozymes. For example, *H. zea* lysozyme is 90% identical to *Heliothis virescens* lysozyme (Shelby et al., 1998) and 76% identical to *M. sexta* lysozyme (Mulnix and Dunn, 1994). A phylogenetic tree was constructed using the Neighbor Joining method based on insect lysozyme amino acid sequences (Figure 3). The *H. zea* lysozyme was grouped together with lysozymes from other lepidopteran insects.

The Expression of H. zea Lysozyme Gene. Northern analysis using total RNA from *H. zea* salivary glands showed that the lysozyme probe hybridized to an mRNA of ~1 kb (Figure 4), which confirmed that the *H. zea* lysozyme was expressed in the salivary glands. The size of the mRNA was consistent with that predicted from RACE analyses.

To determine if *H. zea* lysozyme expression is developmentally regulated, northern blot was performed on labial gland total RNA from larvae of different ages (Figure 5). The results showed that lysozyme gene expression was not detected on day 0 of the fifth-instar, but reached the highest level on day 1 and decreased thereafter.

To investigate whether lysozyme gene expression is affected by diets, dot blot analysis was performed on labial gland RNA from larvae fed on different host plants. The results revealed that the lysozyme mRNA level was the highest from larvae fed on tomato, followed by cotton and tobacco, with larvae fed on artificial diet being the lowest (Figure 6).

Lysozyme Protein Synthesis. Labial gland homogenates from larvae of different ages were separated by SDS-PAGE, and the proteins were stained with Coomassie blue (Figure 7A). The results showed that labial glands of *H. zea* contain a large number of proteins. The protein profiles were similar from day 1 to 3 of the last-instar. When the clearly separated proteins bands were subjected to N-terminal microsequencing, a *ca.* 14 kDa band produced the sequence

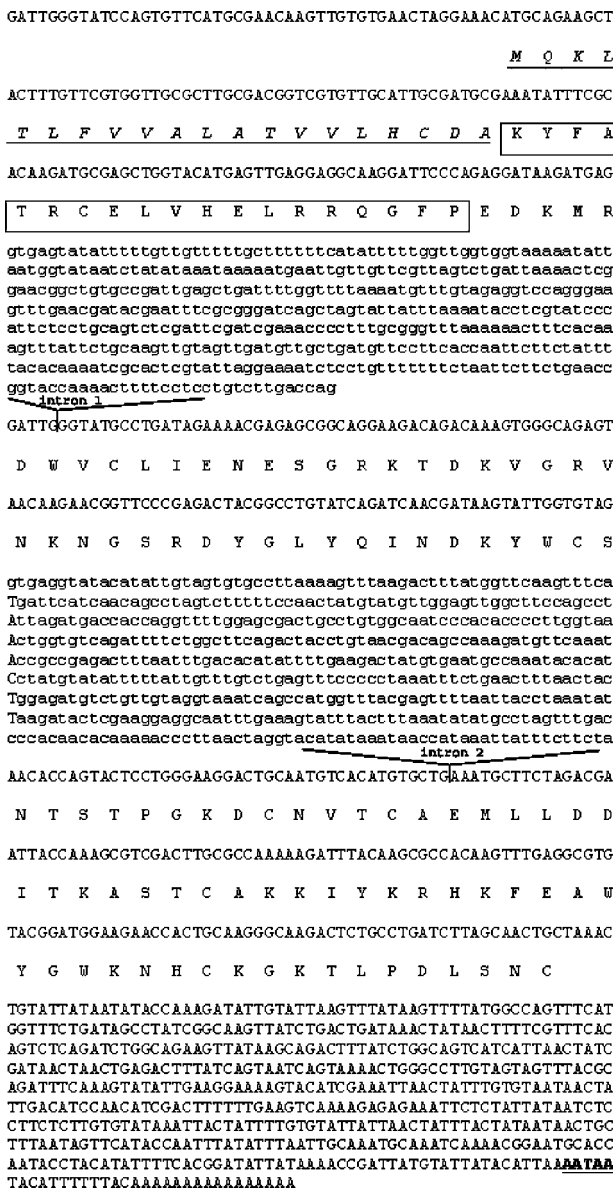


FIG. 1. Lysozyme cDNA and deduced amino acid sequences. The two introns are displayed as DNA sequences in lower case lettering. The N-terminal signal peptide is displayed as amino acid sequence in italic font. The sequenced amino acids are enclosed in a box. The putative polyadenylation signal at the 3′-UTR is underlined and in bold-faced font.

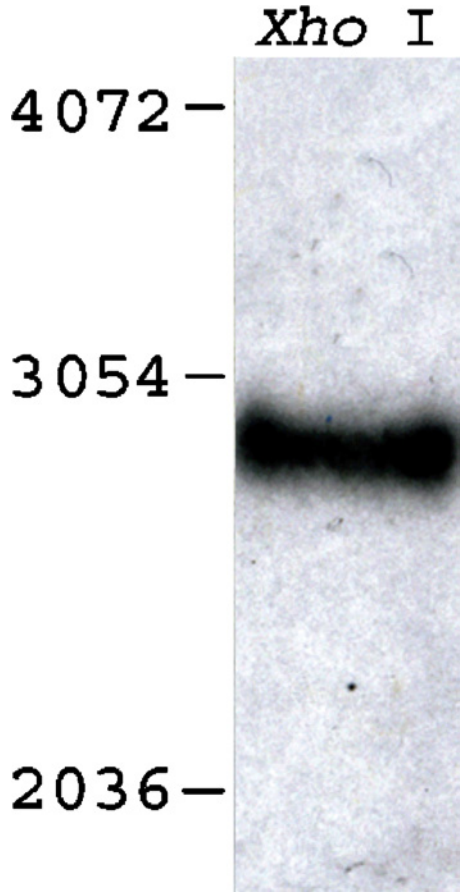


FIG. 2. Southern blot analysis of *H. zea* genomic DNA digested by *Xho*I. The molecular size markers (in bp) are shown on the left.

KYFATRCELVHELRRQGFP, which corresponds to the amino acids 21–39 of the predicted *H. zea* lysozyme. Apparently, the first 20 amino acid signal peptide is cleaved before the mature lysozyme is secreted. It is noteworthy that the 14 kDa lysozyme band was not visible from day 0 labial gland homogenate, which is consistent with the lack of lysozyme mRNA expression on day 0 (Figure 5). Western blot indicated that the anti-*M. sexta* lysozyme antiserum could detect *H. zea* lysozyme. Similar to the results from Coomassie blue staining, lysozyme was detected from day 1 (Figure 7B). The molecular weight of lysozyme is ~14.4 kDa, consistent with the prediction from the cDNA sequence.

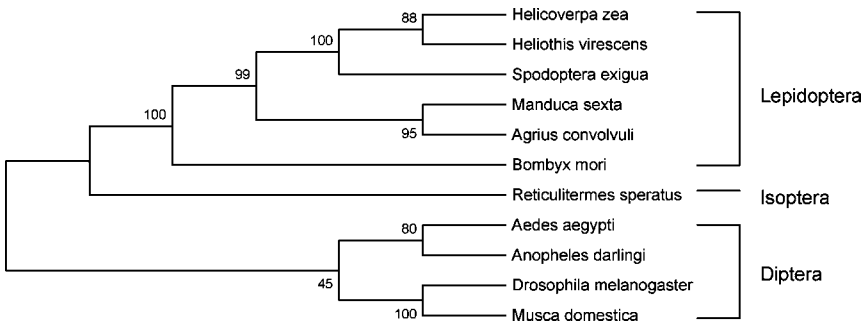


FIG. 3. Amino acid phylogenetic tree of lysozymes among different insects. The tree was constructed by using the neighbor-joining method with 100 bootstrap replications.

We further analyzed the protein profiles in the saliva and regurgitant of last-instar *H. zea* larvae by SDS-PAGE and silver staining (Figure 8). The results showed that the polypeptides in the saliva were very different from those in the regurgitant. Saliva contained *ca.* 10 polypeptides, among which is the abundant lysozyme. In comparison, this band was missing in the regurgitant sample. Similarly, western blot analysis detected lysozyme from saliva and labial glands, but not from regurgitant (Figure 9). Furthermore, two bands were detected in the labial gland samples, and the higher molecular weight band is probably the unprocessed form of lysozyme.

pI Value of H. zea Lysozyme. The pI value of lysozyme was determined by isoelectric focusing using BioRad Rotofor system. Each fraction was concentrated and measured for lysozyme activity. The highest bacterial-lytic activity appeared at the fraction of pH >9.5 (Figure 10).

DISCUSSION

The *H. zea* lysozyme probably belongs to the c-type, because its N-terminus is similar to the c (chicken)-type lysozyme of the other lepidopteran insects (e.g., *Bombyx mori*, *Galleria mellonella*, and *Spodoptera littoralis*) (Jollès et al., 1979). Except for *Drosophila* that possesses multiple copies of unspliced lysozyme genes, other insect lysozyme genes are single-copy genes with at least two introns. Our analysis of the *H. zea* lysozyme demonstrated that it is a single-copy gene and has two introns within the coding region. This finding is consistent with the gene structure for most insect lysozymes (Hultmark, 1996).

The very basic pI value of *H. zea* lysozyme (>9.5) is comparable to *H. virescens* lysozyme (Lockey and Ourth, 1996) and *Hyalophora cecropia* lysozyme

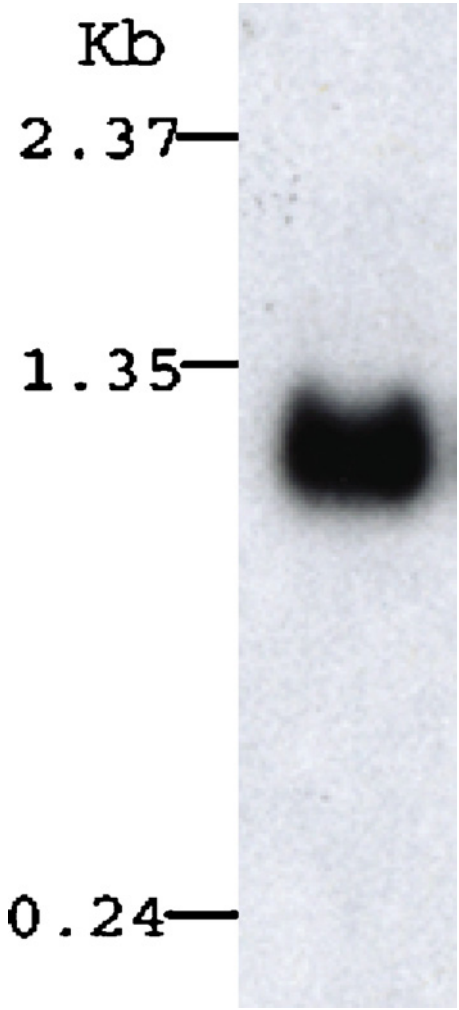


FIG. 4. Northern blot analysis of labial gland total RNA. An aliquot of 10 μg of total RNA were separated on 1% agarose gel and hybridized to the *H. zea* lysozyme cDNA probe. RNA markers are indicated in kb.

(Hultmark et al., 1980), which are both >9.5. A signal peptide of *ca.* 20 amino acids is often present at the N-termini of most lysozymes and cleaved during secretion. Direct sequencing of the mature *H. zea* lysozyme confirmed that it has a 20 amino acid signal peptide. Using an antiserum against *M. sexta* lysozyme, we detected two bands on western blots with *H. zea* labial gland homogenates,

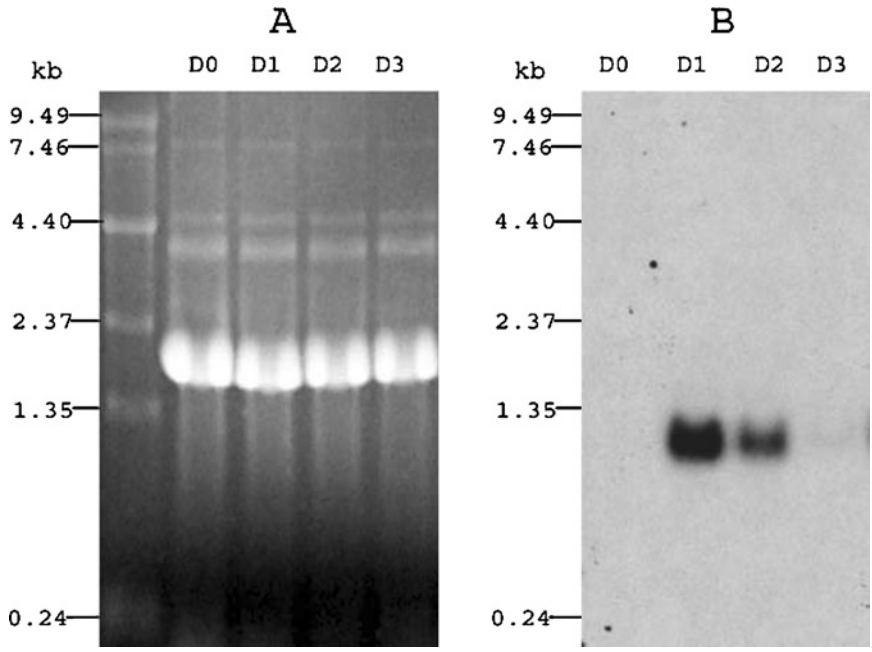


FIG. 5. Temporal expression of lysozyme gene in *H. zea* fifth-instar larvae. (A) An ethidium bromide-stained gel showing uniform intensities of rRNA as the control for equal loading. (B) Northern blot with the *H. zea* lysozyme cDNA probe. D0, D1, D2 and D3 indicate total labial gland RNA from 0, 1, 2, and 3 day-old last-instar *H. zea* larvae.

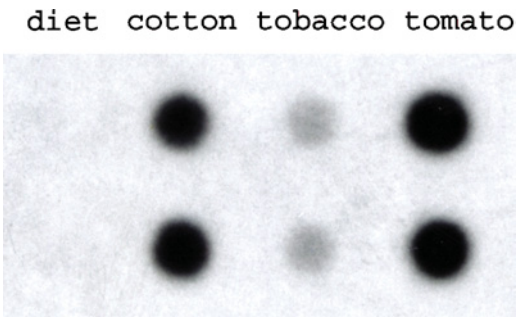


FIG. 6. Dot blot analysis of dietary effects on lysozyme gene expression. Equal amounts (10 μg) of total labial gland RNA were loaded induplicate on a nylon membrane and probed with labeled *H. zea* lysozyme cDNA. Total RNA of labial glands was isolated from day 3 of the last-instar *H. zea* larvae.

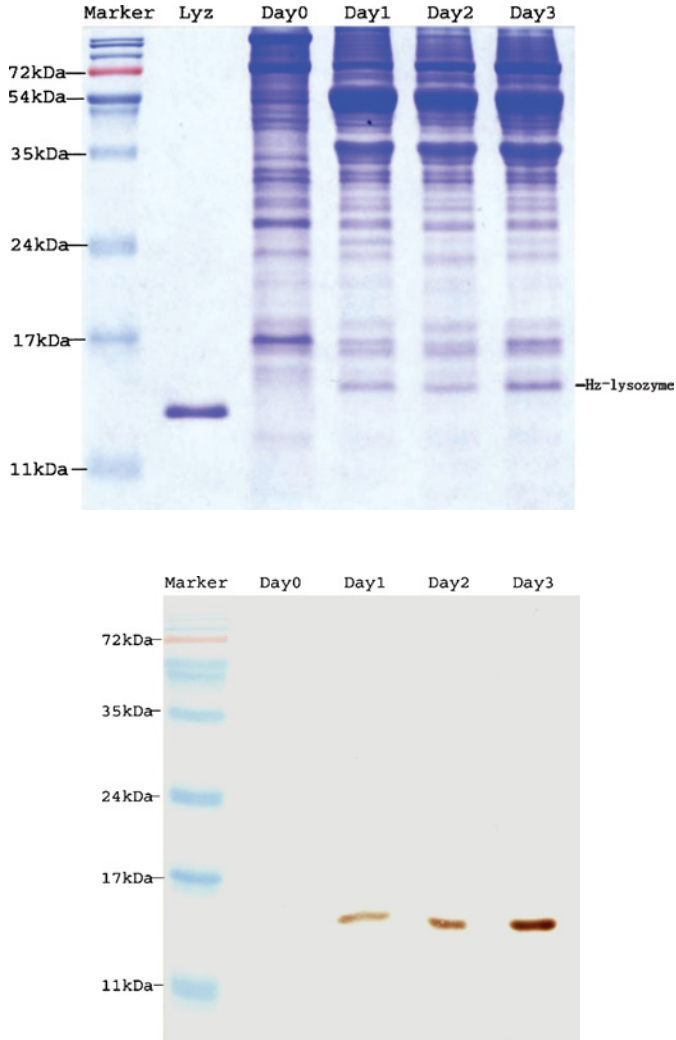


FIG. 7. SDS-PAGE analysis of *H. zea* salivary gland homogenates and western blot. (A) Coomassie staining. Lane 1 shows molecular weight markers in kDa, and lane 2 chicken egg-white lysozyme. Lanes 3–6 show salivary gland homogenates from day 0 to 3 of the last-instar larvae. Each sample was homogenates of 10 pairs of salivary glands. The *H. zea* lysozyme is indicated. (B) Western blot with rabbit anti-*M. sexta* lysozyme antiserum as the primary antibodies. Secondary antibody is horse-radish peroxidase-conjugated anti-rabbit antibody. Lane 1 shows molecular weight markers in kDa, and lanes 2–5 are salivary gland homogenates from day 0 to 3 of last-instar larvae. Approximately 5 μg of each sample were loaded.

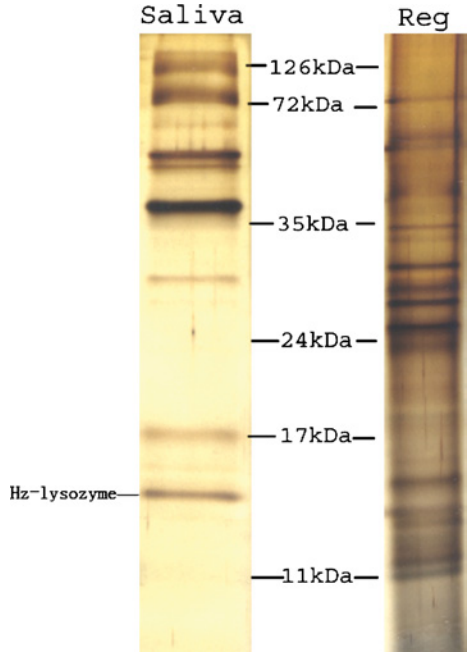


FIG. 8. Protein profiles *H. zea* saliva and regurgitant. Saliva and regurgitant (Reg) were separated by SDS-PAGE and silver-stained. Saliva was collected from 10 last-instar larvae. Regurgitant contained *ca*. 0.5 μg protein.

but not saliva. Dunn et al. (1985) also found two forms of lysozyme in cell-free hemolymph of *M. sexta* by using the same lysozyme antiserum. They identified that the higher MW form was the active enzyme and considered the lower one as a possible degradation product of the lysozyme. In our analysis, we have confirmed the much more intense lower band (14.4 kDa) as the processed mature lysozyme. Although we did not identify the sequence of the higher MW peptide, both its absence in saliva and its similarity in size to the predicted unprocessed lysozyme (16.4 kDa) suggest that it is likely the unprocessed, unsecreted form of lysozyme with the signal peptide still attached.

Lysozyme has been found in various tissues of Lepidoptera, such as fat body, hemocytes, Malpighian tubules, midgut, etc. (Hultmark, 1996). In salivary glands, a very low lysozyme transcript level has been reported for *M. sexta* (Mulnix and Dunn, 1994). In contrast, *H. zea* salivary gland lysozyme is not only at high transcription levels, but also one of the most abundant proteins in saliva. A time course study showed differential expression of *H. zea* lysozyme gene during development, with the highest transcript level being detected on day 1 of

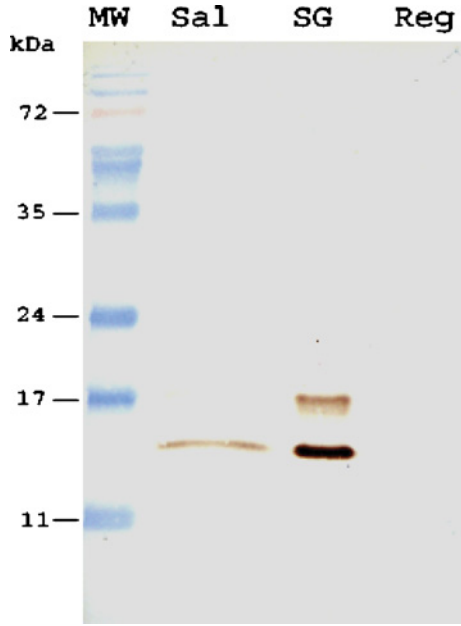
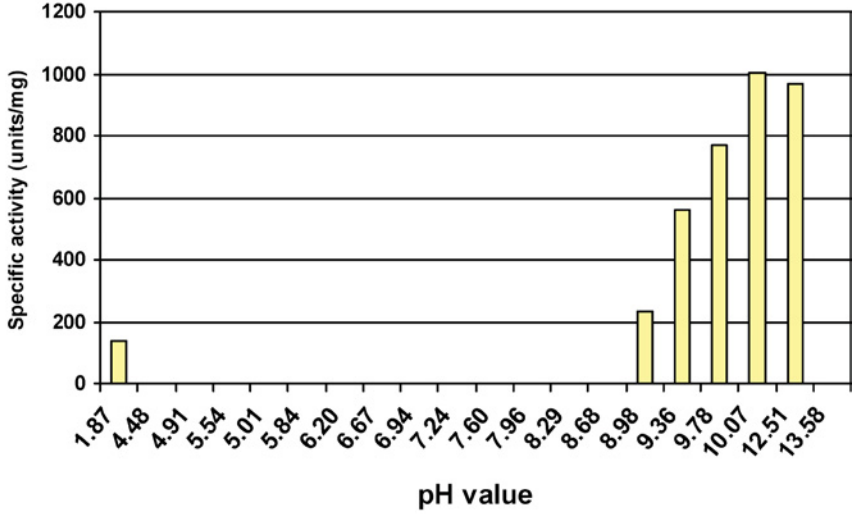


FIG. 9. Western blot analysis of saliva (Sal), labial glands (SG), and regurgitant (Reg). The lysozyme protein was detected with the Vectastain ABC Elite kit (Vector). The amount of protein loaded on each lane was ~5 μg of labial gland homogenates, saliva collected from 20 last-instar larvae, and 2 μg of regurgitant proteins. MW, standard molecular weight marker.

the fifth-instar. This coincides with the beginning of feeding of larvae following the molt, suggesting that the *H. zea* salivary lysozyme plays an important role in antibacterial defense during feeding.

It is intriguing to find that the *H. zea* lysozyme transcription is regulated by the diet, composition, as the larvae fed on artificial diet, and different plants had various lysozyme RNA levels. As far as we know, there are no reports of host plant impact on salivary gene expression. Previous studies only addressed the effect of the salivary components on host signaling pathways or defensive responses. Although we do not know which components in the different plant diets affect the levels of lysozyme induction is not known, it could be speculated that the differential induction is partially related to the bacteria contained in these diets, because lysozyme is inducible by the bacterial cell wall component, peptidoglycan, in most organisms studied. The low transcript level of lysozyme gene from insects fed on artificial diet could be due to antibiotics (streptomycin, aureomycin, and FABCO) in the diet, which may reduce bacterial exposure compared with larvae

Experiment 1:



Experiment 2:

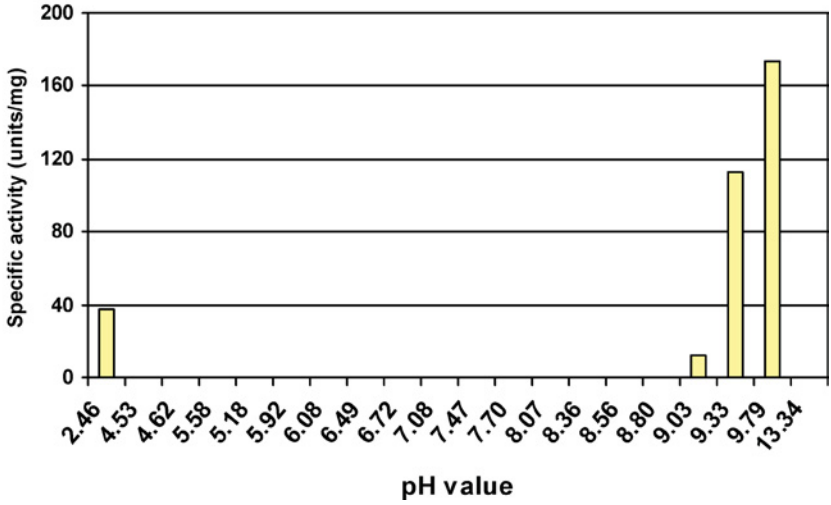


FIG. 10. Lysozyme activity of Rotofor fractions from two experiments for the estimate of pI.

feeding on plants. It should be pointed out that the overall protein concentration of the salivary glands is highest when larvae feed on tobacco, although lysozyme levels are lowest on tobacco-fed larvae. These finding suggests that in larvae fed on different host plants, differential expression of lysozyme may affect their susceptibility to bacteria.

We speculate that the differential expression of lysozyme may play a key role in tritrophic interactions among the host plant, the insect, and the associated bacteria. The plant surface may be inhabited with epiphytic, symbiotic, and/or pathogenic microbes. Lysozyme is secreted extraorally prior to or during ingestion. Bacteria are then lysed on the plant surface, and peptidoglycan fragments and other cell components may then be released. In hemolymph of *Bombyx mori*, there is a 5 hr delay between injection of live bacteria and detection of antibacterial activity (Morishima et al., 1988). Thus, the salivary lysozyme may provide the caterpillar with a pre-ingestive, ready-to-use antibacterial factor. We contend that the saliva of larval lepidopterans has largely been an overlooked factor in their immunity to disease. In addition to lysozyme, saliva also contains significant levels of glucose oxidase, an enzyme noted for its potent antimicrobial activity (Felton and Eichenseer, 1999). Our laboratory is currently characterizing the complex of salivary proteins that are secreted by *H. zea*, and it is likely that many of them play a major role in antimicrobial defense.

Lysozyme may also play a role in eliciting plant-induced responses. Because bacterial cell wall fragments may be released by the action of lysozyme, they may act as elicitors of plant defense. Bacterial cell components contain elicitors that trigger plant defensive responses (Anderson, 1989) such as the salicylic acid (SA) signaling pathway that is involved in defenses primarily against phytopathogens (Hammerschmidt and Smith-Becker, 1999). For plants that harbor symbiotic or epiphytic bacteria, the released elicitors may enter plant tissue through caterpillar's feeding edges and induce the SA signaling of plant defense. Another signaling pathway in plants is the jasmonic acid (JA) pathway, which induces defensive compounds against herbivores and pathogens (reviewed in Walling, 2000). In certain instances of insect–plant systems, the SA and JA pathways produce antagonistic cross-talk (Felton and Korth, 2000). Thus, if elicitors of bacteria induce the SA pathway and indirectly suppress the JA pathway, lysozyme may be yet another tool in their arsenal for suppressing plant defenses.

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JUVENILE HORMONE REGULATES *DE NOVO* ISOPRENOID AGGREGATION PHEROMONE BIOSYNTHESIS IN PINE BARK BEETLES, *Ips* SPP., THROUGH TRANSCRIPTIONAL CONTROL OF HMG-CoA REDUCTASE

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Abstract—Evidence is presented for transcriptional regulation of *de novo* pheromone biosynthesis in *Ips* spp. bark beetles, but the comparative biochemical and molecular approach reveals a dichotomy between species in the *pini* and *grandicollis* subgeneric groups. Radiotracer studies with ^{14}C-acetate demonstrate that feeding on host phloem stimulates biosynthesis in males of three *Ips* spp. However, treatment with juvenile hormone III (JH III) stimulates biosynthesis only in *Ips pini*. Thus, two species in the *grandicollis* subgeneric group (*I. grandicollis* and *I. paraconfusus*) appear to have a different mode of regulation related to JH III than does *I. pini*. Between 16 and 20 hr after feeding has commenced, pheromone production, as measured by accumulation in abdominal tissue, is stimulated about 150- (*I. pini*) and 350-times (*I. paraconfusus*) above the control level of 1–10 ng/male measured at 0 hr. Treatment with JH III results in accumulation in *I. pini* that is 3–4 times more than in phloem-fed males, whereas the identical treatment results in only weak accumulation in *I. paraconfusus* (45-times less than phloem-fed males). Comparative studies of gene expression and enzyme activity related to biosynthesis also support different modes of JH III-related regulation in *I. pini* and *I. paraconfusus*. In

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males of both species, feeding on host phloem results in increased transcript abundance and increased activity for the key *de novo* isoprenoid pathway enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-R). However, while JH III treatment results in comparable maximal increases in HMG-R transcript levels in both species (similar to feeding), the activity of HMG-R in crude extracts from JH III-treated male *I. paraconfusus* is low in comparison with male *I. pini*. Hypothetical explanations for the interspecific dichotomy in the regulation of pheromone biosynthesis include a second hormone or factor in *grandicollis* group species that functions either alone or with JH III; in both cases acting after *HMG-R* has been transcribed.

Key Words—Insects, Coleoptera, Scolytidae, bark beetles, endocrine regulation, corpora allata, isoprenoids, northern blot, mevalonate, *de novo* pheromone biosynthesis, California fivespined ips, *Ips paraconfusus*, pine engraver, *Ips pini*, eastern fivespined ips, *Ips grandicollis*, ipsenol, 2-methyl-6-methylene-7-octen-4-ol, ipsdienol, 2-methyl-6-methylene-2,7-octadien-4-ol, HMG-CoA reductase, juvenile hormone, *cis*-10,11-epoxy-3,7,11-trimethyl-*E*, *E*-2,6-dodecadienoic acid methyl ester.

INTRODUCTION

Pine bark beetles such as the California fivespined ips, *Ips paraconfusus* Lanier, the eastern fivespined ips, *I. grandicollis* (Eichhoff), and the pine engraver, *I. pini* (Say) (Coleoptera: Scolytidae), aggregate in and colonize the phloem of host *Pinus* spp. in response to pheromone blends that include the male-produced C_{10} isoprenoid components, (4*S*)-(–)-ipsenol (2-methyl-6-methylene-7-octen-4-ol) and/or (4*S*)-(+)- or (4*R*)-(−)-ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol) (Silverstein et al., 1966a,b; Vité et al., 1976; Birch et al., 1980). Although the synthesis of these monoterpene alcohols occurs through hydroxylation of the host monoterpene myrcene in *I. paraconfusus* (Hendry et al., 1980; Vanderwel, 1991) and *I. pini* (Vanderwel, 1991), *de novo* pheromone synthesis (via the mevalonate biosynthetic pathway) has been demonstrated through radiolabeling studies with both species (Seybold et al., 1995b; Ivarsson et al., 1997; Tillman et al., 1998).

The regulation of *de novo* monoterpenoid pheromone synthesis has been investigated in *I. pini*, but not in *I. paraconfusus* or *I. grandicollis*. In western North American populations of *I. pini*, where the principal pheromone component is (4*R*)-(−)-ipsdienol (Birch et al., 1980; Seybold et al., 1995a), the *de novo* synthesis of ipsdienol is regulated by the sesquiterpenoid insect developmental hormone, juvenile hormone III (JH III) (Tillman et al., 1998). Feeding on phloem tissue of its predominant host in California, Jeffrey pine, *Pinus jeffreyi* Grev. & Balf., stimulates the corpora allata of males to release JH III [release likely occurs immediately as JH III is biosynthesized (Feyereisen, 1985)], which in turn mediates the stimulation of one or more enzymatically-catalyzed reaction(s),

primarily between acetate and mevalonate, in the isoprenoid pathway (Tillman et al., 1998, 1999; Seybold and Tittiger, 2003).

By analogy to isoprenoid biosynthesis in other insect systems (Gertler et al., 1988; Martinez-Gonzalez et al., 1993; Casals et al., 1996; Duportets et al., 1998; reviewed in Seybold and Tittiger, 2003) and sterol biosynthesis in vertebrate systems (reviewed in Goldstein and Brown, 1990; Hampton et al., 1996), the enzyme 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (HMG-R; E.C. 1.1.1.34) is the primary candidate for a major point of regulation in the isoprenoid pheromone biosynthetic pathway in these scolytids. Other enzymes in the pathway are also likely up-regulated during pheromone biosynthesis in bark beetles (Tittiger et al., 2000; Martin et al., 2003; Keeling et al., 2004), but not to the extent of HMG-R. Based on results from *I. pini*, we hypothesize that JH III is a positive regulator of HMG-R in *Ips* spp. Indeed, in male *I. paraconfusus*, whose principal (i.e., most abundantly produced) pheromone component is (4*S*)-(−)-ipsenol, topical application of JH III increases the levels of the transcript for *HMG-R* in an apparent dose- and time-dependent manner (Tittiger et al., 1999).

In this study, we use biochemical and molecular approaches to examine the comparative regulation of *de novo* biosynthesis of monoterpenoid pheromone components in *I. paraconfusus*, *I. grandicollis* [principal pheromone component is (4*S*)-(−)-ipsenol], and *I. pini* by determining whether JH III interacts with HMG-R at the level of mRNA and/or protein. We describe results from radiotracer analyses of *de novo* ipsenol biosynthesis in phloem-fed or JH III-treated male *I. paraconfusus* and male *I. grandicollis*, and from a complete analysis of total pheromone production, HMG-R activity, and HMG-R transcript abundance in phloem-fed or JH III-treated male and female *I. pini* and *I. paraconfusus*.

METHODS AND MATERIALS

Insects

All three *Ips* spp. were collected as immatures in infested pine logging debris or from standing trees and reared to the adult stage (Browne, 1972). *Ips pini* was collected between 1996 and 1999 in ponderosa pine, *Pinus ponderosa* Laws., or *P. jeffreyi* logging debris from the Lassen National Forest, Lassen Co., CA (Table 1). *Ips paraconfusus* was collected between 1996 and 2000 in either *P. ponderosa,* sugar pine, *P. lambertiana* Dougl. (logging debris), or Monterey pine, *P. radiata* D. Don (standing trees) from the University of California Blodgett Forest, El Dorado Co., CA or from Contra Costa Co., CA (Table 1). *Ips grandicollis* was collected in 2000 from standing trees of Scots pine, *P. sylvestris* L., from two sites in Iowa (Table 1). Beetles were stored at 4°C on moist filter paper or paper toweling after emergence from host material and used within 14 d of emergence. Voucher specimens have been deposited in insect collections at the California Academy

TABLE 1. COLLECTIONS OF *Ips* SPP. FOR BIOCHEMICAL AND MOLECULAR STUDIES OF PHEROMONE BIOSYNTHESIS

Species	Locality and host	Date
Ips pini	CA: Lassen Co.	May, 1996
	1.6 km SE of Halls Flat; T33N, R6E, S24; *Pinus jeffreyi* Grev&Balf & *Pinus ponderosa* Laws.[a]	
	CA: Lassen Co.	Sept., 1996
	~3 km W of Busters Reservoir; T34N, R7E, S15 and 22	
	CA: Lassen Co.	July, 1997
	2 km SE of Harvey Mountain and 1 km S of Barrell Pit Reservoir; T33N, R8E, S13 and T33N, R9E, S18	
	CA: Lassen Co.	August, 1997
	2 km N of Logan Mountain; T32N, R9E, S2 and S4	
	CA: Lassen Co.	June, 1998
	~2 km E. Ashpan Butte; T32N, R4E, S21	
	CA: Lassen Co.	Oct., 1998
	~3.6 km NE Ladder Butte; T34N, R6E, S3	
	CA: Lassen Co.	May, 1999
	~3.6 km E Intersection of Hwys 44&32; T30N, R11E, S34	
	CA: Lassen Co.	May, 2000
	near Blacks Mtn. Exp. Forest; 40°43′03″N, 121°11′11″W	
Ips paraconfusus	CA: El Dorado Co.	Sept., 1996
	Blodgett Research Forest (BRF) near Bakke Ranch Road; T12N, R12E, $NE^1/_4NW^1/_4S9$	
	Pinus lambertiana Dougl. (logging debris)	
	CA: Contra Costa Co.	Dec., 1996
	Briones Reservoir (eastern side); 37°55′21″N, 122°12′4″W; *Pinus radiata* D. Don (standing)	
	BRF	Sept., 1996
	T12N, R12E, $NW^1/_4NW^1/_4S16$	July, August,
	P. ponderosa (logging debris)	Dec., 1997
	BRF	May, 1998
	~1 km N. Kings Meadows,~1200 m elev.	Oct., 1998
	T12N, R12E, S17; T12N, R12E, S24	June, 1999
	P. ponderosa (logging debris)	
	BRF	May, 2000
	near Gaddis Creek, ~1200 m elev.; 38°35′19″ N, 120°38′47″ W; *P. ponderosa* (logging debris)	
Ips grandicollis	IA: Polk Co.	July, 2000
	Jester State Park, near Polk City; *Pinus sylvestris* L. (standing)	
	IA: Story Co.	August, 2000
	near Veterinary Medical Research Complex; Iowa State University, Ames; 42°N, 93°W; *P. sylvestris* (standing)	

[a] All populations of *Ips pini* were collected in *Pinus jeffreyi* or *Pinus ponderosa* logging debris at approximately 2000 m elevation.

of Sciences (San Francisco, CA, USA) and the Canadian National Collection (Ottawa, Ontario, Canada).

Chemicals and Materials

Since this study involved a broad range of bio-organic, biochemical, and molecular techniques, a wide array of reagents and materials were used (Table 2).

Experimental Treatments

Feeding on Host Phloem. For radiolabeling studies of *de novo* pheromone biosynthesis, *in vitro* assays of HMG-R activity, and northern blot analyses of HMG-R transcript abundance, phloem for feeding *I. pini* (fed on *P. jeffreyi*), *I. paraconfusus* (fed on *P. ponderosa*), or *I. grandicollis* (fed on *P. sylvestris*) was prepared as previously described (Tillman et al., 1998). Coiled strips of pine phloem in plastic 35 mm photo film canisters were ventilated (to prevent growth of molds and asphyxiation of beetles) by cutting an ~20 mm diam hole in the canister lid and covering the hole with aluminum screening. Insects (25–35 in each canister) were allowed to feed for specified times. Males were fed for 48 hr for radiolabeling studies of *de novo* pheromone biosynthesis, and both males and females were fed for 0, 6, 12, 16, 20, and 26 hr for *in vitro* assays of HMG-R activity. For northern blot analyses of HMG-R, male and female *I. pini* were fed for 0, 16, 24, 48 and 72 hr, and male *I. paraconfusus* were fed for 0 and 20 hr. Insects were treated and then frozen at −80°C until the RNA was isolated. For radiolabeling studies of *de novo* pheromone biosynthesis, the phloem-fed samples were replicated four times (a group of 14 males/replicate). For *in vitro* assays of HMG-R activity, 40–60 insects were fed on phloem and then the group was homogenized, split into three samples, and each sample was assayed independently. For northern blot analyses of HMG-R, samples were replicated three times (a group of 8–16 insects/replicate).

For studies assessing pheromone accumulation in abdominal tissue in phloem-fed *I. pini* or *I. paraconfusus*, males were inserted into predrilled holes in the phloem of cut logs for 0, 4, 8, 12, 16, 20, 28, and 36 hr (Seybold et al., 1995a; Lu, 1999). Aluminum screening strips were stapled over holes to retain inserted beetles. Insects were removed from logs by peeling fresh phloem off in strips. Treatments were replicated six times (a group of 30 males/replicate) for each time period.

Application of Juvenile Hormone III. For radiolabeling studies of *de novo* pheromone biosynthesis in male *I. paraconfusus* and *I. grandicollis*, 10 μg of JH III dissolved in 0.5 μl acetone were applied topically to the ventral abdominal cuticle of each insect with a 10 μl syringe with a 33-gauge, blunt-tipped needle

TABLE 2. CHEMICALS, REAGENTS, AND OTHER MATERIALS FOR BIOCHEMICAL AND MOLECULAR STUDIES OF PHEROMONE BIOSYNTHESIS IN *Ips* SPP.

Product name	Manufacturer	Catalog number
Bio-organic standards/reagents/solvents		
Acetone	Fisher Scientific, Pittsburgh, PA (F)	A949-4
4-Decanone	Aldrich Chemical Co., Milwaukee, WI (Al)	19,467-0
Diethyl ether	F	E138-1
EDTA (disodium salt)	Sigma Chemical Co, St. Louis, MO (Si)	E-1644
Hexane	F	H303-4
Ipsdienol	Bedoukian Research Inc., Danbury, CT (B)	P407
Ipsenol	B	P408
Juvenile hormone III	Si	J2000
Pentane	Mallinckrodt Specialty Chemicals Co., Paris, KY	6145-4
Sucrose	United States Biochemical Co., Cleveland, OH (U)	21938
Biochemical reagents/materials		
Bovine serum albumin	Si	A-4503
Dithiothreitol	Si	D-5545
Ecolite(+) biodegradable liquid scintillation cocktail	ICN Biomedicals, Costa Mesa, CA (I)	882475
Florisil (60/100 mesh)	Supelco Chrom. Products, Bellefonte, PA (Su)	20280
Hydrochloric acid	F	A144-212
[^{14}C]Hydroxymethylglutaryl-CoA[a]	NEN Life Science Products, Boston, MA (N)	NEC-642
[^{14}C]Mevalonolactone (TLC standard)[a]	N	NEC-679
(*R*)-(+)-1-(1-Naphthyl)ethylamine	Al	23,744-2
NADPH	Si	N-1630
Porapak Q (50/80 mesh)	Su	20339
Potassium chloride	F	P217-500
Potassium phosphate	F	P288-500
Silica-coated paper (TLC)	Gelman Scientific, Ann Arbor, MI	61886
Sodium [1-^{14}C]acetate[a]	I	12013
Sodium sulfate	F	BP354-500
Molecular reagents/materials		
Agarose	GIBCO-BRL Life Technologies, Bethesda, MD (G)	15510-027
pBluescript KS+ cloning vector	Stratagene, La Jolla, CA (St)	212207
Bromochloroform	Molecular Research Center, Cincinnati, OH	BP-151
Bromophenol blue	U	12370
dATP (unlabeled)	G	10216-018
dGTP (unlabeled)	G	10218-014

TABLE 2. CONTINUED

Product name	Manufacturer	Catalog number
dTTP (unlabeled)	G	10219-012
[^{32}P]DeoxyCTP[a]	N	BLU-013H
DMSO	Si	D-5879
Formamide	F	BP227-500
Glyoxal	F	BP1370-500
Hybond N nylon membranes	Amersham Pharmacia Bio., Piscataway, NJ (Am)	RPN1510N
Iodoacetate	F	Acros #17097-0250
50 mM Magnesium chloride	G	Y02016
Mouse β-actin cDNA	St	200407
PCR primers	G	not applicable
PCR 10X buffer	G	Y02028
RNA markers	Novagen, Madison, WI (N)	69946
SDS	F	BP166-100
Sephadex G-50	Am	17-0045-02
Sodium citrate	F	BP327-1
Sodium chloride	F	BP358-212
Sodium phosphate	F	S373-500
Taq Polymerase	G	10342-020
TRIzol reagent	G	15596-026
pT7Blue plasmid cloning kit	N	69901-3
Xylene cyanol FF	U	23513

[a] Specific activities: HMG-CoA: 56.98 mCi/mmole; Mevalonolactone: 40–60 mCi/mmole: Sodium Acetate: 65 mCi/mmole; DeoxyCTP: 3000 Ci/mmole, depending upon lot.

(Hamilton Company, Reno, NV). The dose was adjusted for the chemical purity of the commercial material. Control applications consisted of topical treatment with 0.5 μl acetone. Treated males were incubated for 12–16 hr on moist glass wool in 250 ml glass storage bottles (Lu, 1999). Treatments were replicated four times (a group of 14 males/replicate). Following incubation, insects were injected with sodium [1-^{14}C]acetate (see below).

For studies measuring pheromone accumulation in abdominal tissue (total pheromone production) in JH III-treated males, individual *I. pini* and *I. paraconfusus* were treated topically as described above with 0.5 μl of an acetone solution containing: (1) 5.84 μg JH III (for time curve) and incubated for specified time periods (0, 4, 8, 12, 16, 20, 28, or 36 hr); or (2) specified doses of JH III (0, 0.06, 0.6, 1.0, 6.0, 10.0, or 100.0 μg per insect) and incubated for 20 hr (for dose curve). Doses of JH III were adjusted for chemical purity of the commercial material. After JH III treatment, insects were incubated as described above (Lu, 1999). Treatments were replicated 6–12 times (a group of 30 males/replicate) for each JH III time period or dose. Following incubation, pheromone was extracted from abdominal tissue and quantified by gas chromatography with a flame ionization

detector (GC-FID) (see below). Incubation times and doses involving pheromone production were based on previous studies of JH III or its analogues with these *Ips* species (Chen et al., 1988; Tillman et al., 1998).

For *in vitro* assays of HMG-R activity and northern blot analyses of HMG-R transcript abundance, male or female *I. pini* and *I. paraconfusus* were treated topically as described above with 0.5 μl of an acetone solution containing: (1) 10.0 μg JH III (for time curve) and incubated for specified time periods (0, 6, 12, 16, 20, or 26 hr); or (2) specified doses of JH III (0, 0.1, 1.0, 10.0, 100.0, or 200.0 μg per insect) and incubated for 20 hr (for dose curve). The doses of JH III were adjusted for the chemical purity of the commercial material. Treated insects were incubated in glass jars with cotton-based packaging string wound in the bottom to provide a tactile medium for locomotion. A 7 ml glass vial filled with distilled water was suspended near the top of the jar to maintain humidity. Incubations occurred in the dark to simulate the photo-condition encountered when *Ips* spp. feed under bark tissue. For assays of HMG-R activity, treatments were applied once (a group of 30–60 male or female insects) for each JH III time period or dose, and the crude microsomal fraction extract was assayed separately three times. One assay replicate from each of three conditions in the female *I. paraconfusus* JH III dose experiment was omitted due to sample contamination that occurred during postassay sample handling. These specific replicate omissions are indicated below (in Results). The entire male *I. paraconfusus* JH III dose experiment measuring HMG-R activity was repeated to confirm the initial finding. For northern blot analyses of HMG-R, the JH III-treated time and dose samples were replicated 3 times (a group of 7–15 insects/replicate). Insects for HMG-R northern analyses were treated and stored at -80°C until the RNA was isolated.

Administration of Sodium [1-^{14}C]Acetate for Study of de novo Pheromone Biosynthesis. Following acetone treatment and incubation for 12–16 hr (control), JH III treatment and incubation for 12–16 hr, or feeding on host phloem for 48 hr, individual males of *I. paraconfusus* or *I. grandicollis* were injected between the abdominal sternites (Seybold et al., 1995b; Tillman et al., 1998) with $\sim$0.2 μCi sodium [1-^{14}C]acetate (65 mCi/mmol) dissolved in 0.5 μl saline (0.15 M aqueous sodium chloride) with a 10 μl syringe with a 33-gauge, beveled-tip needle (Hamilton Company, Reno, NV).

Sample Preparation

De novo Pheromone Biosynthesis. The protocol and experimental design for the measurement o*f de novo* pheromone biosynthesis in *I. paraconfusus* and *I. grandicollis* males were identical with the study of male *I. pini* by Tillman et al. (1998). This experiment was conducted twice for each species: *I. paraconfusus*—21–24 and 23–26, July 2000 and *I. grandicollis*—27–30, August 2000 and 19–22,

September 2000. Samples from *I. paraconfusus* and *I. grandicollis* were prepared by trapping volatiles containing ^{14}C-labeled pheromone released into the headspace by feeding males that had been previously injected with ^{14}C-acetate. Prior to injection, these insects had been either: (1) treated with acetone (control) or JH III and incubated for 12–16 hr; or (2) fed on host phloem for 48 hr. After ^{14}C-acetate injection, each insect was immediately inserted into a predrilled (approximately 3 mm diam) hole in a phloem/bark strip for volatile pheromone collection. A *P. ponderosa* or *P. sylvestris* log was shaved until a thickness of 3–5 mm of phloem and bark remained. Strips of approximately 30 × 280 mm were cut from the shaved log. After the males were inserted into individual holes in the strip, aluminum screening was stapled to both the phloem and bark surfaces to secure the insects. Phloem/bark strips containing treated beetles were then placed into 50 × 305 × 25 mm plexiglass aeration chambers (designed by D.R. Quilici, University of Nevada, Reno, unpublished). Volatiles released during the ensuing 72 hr feeding and pheromone production period by each group of 14 males (one replicate) were trapped on 0.8 g Porapak Q (Byrne et al., 1975) at an air flow rate of 100 ml/min. Porapak Q was extracted in a glass column with 150 ml pentane by gravity flow. An internal standard (80.8 μg of 4-decanone) was added to the pentane extract, which was concentrated to 2 ml by Kuderna-Danish evaporative concentration. Ipsdienol and ipsenol in the samples were quantified by GC-FID (see below).

Accumulation of Pheromone in Abdominal Tissue (Total Pheromone Production). Upon completion of designated feeding or incubation times, samples of abdominal tissue from *I. pini* and *I. paraconfusus* were prepared by dissecting the midgut, hindgut, and associated alimentary and peri-alimentary tissue from individual males and combining the tissue for each replicate into 5 ml vials containing 2 ml pentane:diethyl ether (1:1, v:v) along with 4-decanone (80.8 μg/sample). In *Ips* spp., the anterior midgut is the site of pheromone synthesis (Hall et al., 2002), and the midgut and hindgut are the sites of pheromone accumulation (Byers, 1983). Tissue was homogenized with a blunt-ended stirring rod, vortexed, and sonicated in a water bath for 2–3 min. Extracts were loaded onto a Pasteur pipette containing sodium sulfate (upper layer) and Florisil (4:2 cm). Wash solvent generated after abdominal tissues that had been rinsed three times in the original vial was also loaded onto the column. The extracts were concentrated to 100–200 μl for GC-FID analysis by free evaporation of solvent at room temperature.

In vitro HMG-CoA Reductase Activity. Crude microsomal fractions for *in vitro* assays of HMG-R (HMG-R protein is located in the endoplasmic reticulum) were prepared by serial centrifugation. We modified the assays of Goldstein et al. (1983) and Casals et al. (1996) for use with fed or JH III-treated male or female *I. pini* and *I. paraconfusus*. Assay conditions of HMG-CoA concentration,

microsomal total protein concentration, pH, and assay incubation time and temperature were optimized for HMG-R activity using microsomal fractions from male *I. pini* prior to experimental assays.

Treated insects were anesthetized on ice, and whole body tissue was macerated in 10 ml homogenization buffer [100 mM sucrose, 40 mM potassium phosphate, 30 mM EDTA (disodium salt), 50 mM potassium chloride, and 10 mM dithiothreitol, pH 7.2] with a Polytron® homogenizer (Brinkmann Instruments, Westbury, NY). Microsomal fractions were partitioned from crude tissue homogenates by serial centrifugation on a Beckman J2-21M/E at 4°C (tissue homogenate centrifuged at 7740 × *g* for 5 min; resulting supernatant centrifuged at 12,100 × *g* for 20 min). To ensure complete removal of mitochondria, the supernatant resulting from the 12,100 × *g* spin was further centrifuged at 46,000 × *g* for 15 sec. The microsomal fraction was pelleted from this supernatant by high speed centrifugation on a Beckman L8-55 ultracentrifuge (165,000 × *g* for 60 min at 4°C). Microsomal pellets were re-suspended in approximately 200 μl assay buffer [50 mM potassium phosphate, 5 mM dithiothreitol, 5 mM ETDA (disodium salt), and 200 mM potassium chloride, pH 7.4]. Total microsomal solution protein concentration was determined by the method of Bradford (1976) with bovine serum albumin (1 mg/ml) as an external standard.

HMG-CoA Reductase Transcript Abundance. Total RNA from JH III-treated insects was isolated by TRIzol® reagent (phenol/guanidine isothiocyanate) according to the manufacturer's protocol from pretreated, frozen (−80°C) insects. Total RNA or mRNA could not be isolated consistently from fed insects using TRIzol® reagent or any of numerous other commercial or research protocols that we applied. RNA isolation from coniferous tissue is technically difficult due to uncommonly high titers of lignins, polysaccharides, and/or polyphenols typical of this tissue (Kiefer et al., 2000). We speculate that coniferous tissue present in alimentary canal of fed insects was responsible for these technical difficulties. Nonetheless, in two preliminary studies, RNA samples for northern analyses from fed insects were prepared. Male or female *I. pini* were fed on *P. jeffreyi* phloem for 0, 16, 24, 48 and 72 hr, and male *I. paraconfusus* were fed on *P. ponderosa* phloem for 0 and 20 hr. Treatments for the *I. pini* time course were not replicated, and a complete time course for *I. paraconfusus* was not performed, but in the latter case, the treatments were replicated three times.

Sample Analysis

De novo Pheromone Biosynthesis. Levels of *de novo* pheromone biosynthesis were determined by measuring the quantity of radiolabeled pheromone (ipsenol and/or ipsdienol) in volatiles trapped from the headspace of treated, feeding insects. Total (radiolabeled and unlabeled) production of pheromone was quantified

in some of these samples by GC-FID using a Shimadzu GC-17 gas chromatograph (GC). An HP-Wax column [60 m × 0.25 mm (i.d.), 0.25 μm film thickness] was utilized for separation (Hewlett-Packard Company; San Fernando, CA). The GC was temperature programmed from 60°C to 220°C at 2°/min. Injector and detector were set at 220°C and 240°C, respectively. The flow rate of carrier gas (helium) was 31 cm/sec. Monoterpene alcohols in crude (unfractionated) extracts were quantified by comparing the ratio of the specific monoterpene alcohol peak area to the 4-decanone (internal standard) peak area by using calibration curves. Calibration curves were constructed by plotting the $\log_{10}$ of the ratio of area of monoterpene alcohol to area of 4-decanone vs. $\log_{10}$ mg of standard monoterpene alcohol.

Following quantitative analysis, three 100 μl aliquots were removed from each pentane extract. These aliquots were analyzed on a Beckman LS-6500 liquid scintillation analyzer (LSA) using Ecolite scintillation cocktail to determine the total level of ^{14}C-associated radioactivity in each crude extract. The ^{14}C counting efficiency was 97%.

An aliquot of each concentrated pentane extract was fractionated by high pressure liquid chromatography (HPLC) using a Shimadzu HPLC system, including an SCL-10A *VP* system controller and an LC-10AD *VP* liquid chromatograph. Separations were performed with a 10 mm × 50 cm Nucleosil 50-5 normal-phase column (Alltech Associates, Inc., Deerfield, IL). Solvent system was hexane:acetone (96:4, vol:vol) at a flow rate of 2 ml/min, and mass detection was by UV (235 nm). The volume of the aliquot was adjusted to provide a sample load of approximately 10,000 dpm of ^{14}C-associated radioactivity. Fractions (every 30 sec) were collected with Pharmacia LKB-FRAC-100 Fraction Collector (Pharmacia Biotech, Uppsala, Sweden). Each fraction was assayed by LSA, and the presence of the ipsenol or ipsdienol peak was verified by a comparison of sample HPLC retention times with those of reference standards. The quantity of ^{14}C-associated radioactivity in the ipsenol or ipsdienol fractions from each sample aliquot was adjusted by the appropriate volume factor to yield the total ^{14}C-associated ipsdienol or ipsenol in each original 2 ml sample.

A previous study of *I. pini* and *I. paraconfusus* (Seybold et al., 1995b) validated the same pheromone isolation and HPLC fractionation techniques. The presence of ^{14}C-ipsenol or -ipsdienol in HPLC fractions was established by comparison of HPLC and GC retention times with standards as well as by HPLC and gas chromatography-mass spectrometric (GC-MS) analyses of diastereomeric derivatives of ipsenol (α-methoxy-α-(trifluoromethyl)phenylacetic acid [MTPA] ester) and ipsdienol (camphanic acid ester). Thus, we are confident of our assay for ^{14}C-ipsenol or -ipsdienol in the current study.

Accumulation of Pheromone in Abdominal Tissue (Total Pheromone Production). Levels of total pheromone production were determined by measuring

the quantity of pheromone accumulated in abdominal tissue of male *I. pini* and *I. paraconfusus* that had been fed on host phloem or treated with JH III. Pheromone released by the beetles into the volatile headspace during the feeding or incubation period was not quantified. All crude, concentrated abdominal tissue extracts were analyzed quantitatively for ipsdienol, ipsenol, amitinol and/or *E*-myrcenol using GC-FID and the internal standard technique noted above.

In vitro HMG-CoA Reductase Activity. Levels of *in vitro* HMG-R activity were determined by measuring the quantity of radiolabeled enzyme product isolated from the reaction mixture containing the re-suspended crude microsomal pellet (and HMG-R enzyme) from treated insects. [^{14}C]3-Hydroxy-3-methylglutaryl-CoA ([^{14}C]HMG-CoA) enzyme substrate (56.98 mCi/mmol) was diluted with unlabeled HMG-CoA to attain a working solution with approximately 225,000 dpm ($\sim$0.1 μCi) and 35 nmol total (labeled and unlabeled) HMG-CoA per 5 μl (quantity added to each assay sample). Assay concentrations of 175 μM total HMG-CoA, 10 mM NADPH, and 0.5 mg/ml microsomal protein were combined in 200 μl assay buffer. After a 10 min reaction incubation at 37°C in a shaking water bath, reactions were terminated by the addition of 20 μl of 5 M HCl. Acidified samples were incubated an additional 30 min to allow acid-catalyzed conversion of mevalonate to a cyclic derivative, mevalonolactone (MLL). This conversion facilitated silica paper chromatographic (SPC) separation of unreacted HMG-CoA from the enzyme product (mevalonate). Five milliliters of diethyl ether followed by $\sim$0.5 g of sodium sulfate (Na_2SO_4) were added to the aqueous reaction medium. The medium was extracted with a total of three 5 ml ether aliquots and combined over Na_2SO_4. Combined ether extracts were concentrated to approximately 200 μl by free evaporation at room temperature. Silica paper chromatography (SPC) was used to separate [^{14}C]mevalonolactone from residual unreacted [^{14}C]HMG-CoA in a solvent development system of 1:1 hexane:acetone. A MLL standard was run with every SPC development. The migrations (R_f values) of all radiolabeled SPC compounds were determined via analysis on a BioScan Imaging Scanner System 200-IBM (BioScan, Inc., Washington, D.C.). The R_f value of [^{14}C]mevalonolactone assay product was estimated by comparison to the R_f value of the MLL standard. The silica paper containing radiolabeled MLL was manually excised and the radioactivity quantified by LSA using a Beckman LS-6500 liquid scintillation analyzer as described above.

A derivative of the enzyme assay product (mevalono-[α-(1′-naphthyl)ethyl] amide = MNE-amide) was prepared using (*R*)-(+)-1-(1-naphthyl)ethylamine reagent (NE-amine) (Bergot et al., 1979) and subjected to Fast Atom Bombardment GC-Mass Spectrometry (FAB-GC-MS) to provide additional confirmation that the assay product was MLL. Assay product (from combined samples representing approximately 300 male *I. pini* from *P. jeffreyi* phloem-fed and JH III treatments) was isolated by SPC as described and de-absorbed from silica coating

with a single 30 ml ether extraction of excised silica paper. Extract was filtered through a glass wool-plugged Pasteur pipette to remove suspended silica particles, and concentrated to approximately 200 μl under a gentle nitrogen stream. The relatively small yield of MLL assay product made this derivatization reaction technically difficult. Thus, 1.25 mg of unlabeled, exogenous MLL standard was added prior to derivatization to ensure that the reaction went to completion. MNE-amide was also prepared from labeled MLL (supplemented with unlabeled MLL) for use as a standard. For the derivatization reaction, approximately 17.4 mg NE-amine (approximately 10 $\times$ NE-amine molar excess) were added to the MLL and incubated at 45°C for approximately 4 hr (Bergot et al., 1979) with intermittant vortexing. One milliliter of 1 M HCl was added to the reaction mixture, followed by serial extraction with five 3 ml ether aliquots. The MNE-amide derivative was isolated from the concentrated extract by SPC, extracted from silica paper, and concentrated as described above. Concentrated extract from the assay product sample was passed through a 0.45 μM filter and analyzed by FAB-GC-MS at the University of Nevada Mass Spectrometry Facility for identification by comparison with MNE-amide standard.

HMG-CoA Reductase Transcript Abundance. Levels of HMG-R transcript were determined by northern blot analysis of total RNA from phloem-fed or JH III-treated male or female *I. paraconfusus*. Messenger RNA was analyzed from phloem-fed *I. pini*; total RNA was analyzed from JH III-treated *I. pini*. Radiolabeled DNA probes for northern hybridizations with separated RNA from *I. pini* and *I. paraconfusus* were prepared as follows. 3′ RACE (Rapid Amplification of cDNA Ends) utilizing a degenerate primer (PARAHMD) (Tittiger et al., 1999) and CSX primer yielded approximately 1.2 kb PCR products from first strand cDNA templates prepared (Superscript II reverse transcriptase, Life Technologies) from polyadenylated RNA isolated (QuickPrep micro spin columns, Pharmacia) from JH III-treated male *I. pini* (Hall et al., 2002) and *I. paraconfusus* (Tittiger et al., 1999), respectively. The *I. pini* HMG-R partial 1.2 kb cDNA was cloned into the EcoRV site of a pT7Blue vector. This construct was used as a template to synthesize a radiolabeled HMG-R probe (approximately 430 bp fragment spanning nucleotides 2470–2899 of the partial length cDNA) for northern hybridizations with *I. pini* RNA. Full length cDNA sequence for *I. pini* has been deposited in GenBank (AF304440) (Hall et al., 2002). The 1.2 kb cDNA fragment from *I. paraconfusus* was used directly as a template to synthesize a radiolabeled HMG-R probe for northern hybridizations with *I. paraconfusus* RNA (approximately 960 bp fragment spanning nucleotides 986–1944, which consisted of sequence in both the coding and membrane anchor regions of HMG-R). Full length cDNA sequence for *I. paraconfusus* is deposited in GenBank (AF071750) (Tittiger et al., 1999). HMG-R probes for both *I. pini* and *I. paraconfusus* were labeled with ^{32}P-deoxy-CTP in separate PCR reactions (Mertz and Rashtchian, 1994). Sequence-specific

primers annealing to internal HMG-R cDNA sequence were used for the PCR labeling reactions. Mouse actin cDNA (for use as a gel loading control) in pBluescript vector was similarly labeled using standard T3/T7 primers annealing to respective vector RNA polymerase promoter regions. Following the labeling reactions, unincorporated radio-nucleotides were removed from the cDNAs by centrifugation through Sephadex G-200 columns in TNE buffer (10 mM tris pH 8.0, 1 mM EDTA, 150 mM NaCl) (Tittiger et al., 2003).

For northern analyses, RNA (20 μg/lane) was separated on a glyoxal agarose gel (Sambrook et al., 1989), transferred to Hybond N nylon membrane, and immobilized by ultraviolet crosslinking on a UV Stratalinker 1800 (Stratagene, La Jolla, CA) followed by heat drying for 2 hr at 80°C. Membranes were hybridized with ^{32}P-HMG-R probes followed by separate hybridization with mouse actin to control for potential variability in gel loading. Both male and female northern blots for each experiment were hybridized with the same quantity of the same probe solution. Membranes were washed (Sambrook et al., 1989) under stringent conditions following HMG-R hybridization [(two washes (100 ml) for 20 min at room temperature with 2 × SSC, 0.1% SDS and one wash (100 ml) for 30 min at 67°C with 0.2 × SSC, 0.1% SDS] and under moderately stringent conditions following actin hybridization [two washes (100 ml) for 20 min at room temperature with 2 × SSC, 0.1% SDS and one wash (100 ml) for 30 min at 42°C with 0.2 × SSC, 0.1% SDS]. Membranes were imaged on a BioRad (Hercules, CA) Molecular Imager, and densitometric analyses were performed using Molecular Analyst software (BioRad) (Tittiger et al., 1999, 2003). Densitometric analysis is a computer technique utilized to convert gel lane band digital intensity into numerical values, which correspond to the quantity of mRNA of interest in each gel lane. Expression levels were normalized first to the amount of actin RNA in each lane (to control for variances in gel loading), and then to the expression level (within a sex) of the normalized control treatment for each experiment or data set. In each case, the mean signals for the controls (0 hr feeding, 0 hr post-JH III incubation, or 0 μg JH III) were arbitrarily set to 1.

Data Handling and Statistical Analyses

Masses of pheromone produced were expressed in ng/male, quantities of radiolabeled pheromone produced were expressed in nCi/sample of 14 males, activity of HMG-R was expressed as %nmoles of HMG-CoA converted/sample of a group of 40–60 males or females, and HMG-R transcript abundance was expressed in units of relative expression/sample of 7–16 males or females. For all time course and dose studies, results were plotted regarding time or dose at evenly spaced intervals. Doses of JH III were plotted as $\log_{10}$ [μg JH III + 1].

All data analyses were performed by using SPSS for Windows version 6.1 (SPSS, 1994). Differences in incorporation of ^{14}C-acetate into ipsenol and

ipsdienol between phloem-fed, JH III-treated, and control groups of beetles were tested using one-way analysis of variance of square-root-transformed data for each pheromone component. Pairwise comparisons between the three treatments were tested using Least Significant Difference tests with α controlled experimentwise and set at 0.05 (SPSS, 1994).

Differences in responses in all time course and dose studies were tested by using analysis of variance, and trends in the data were tested with polynomial contrasts. For these analyses, each time period or dose was regarded as independent from others in the test (groups of beetles were sampled destructively at each time period), and the progressions of time periods or doses were regarded as equally spaced levels. If the response behaved in a simple time- or dose-dependent manner, then the linear contrast in the analysis was significant. If the linear contrast was not significant, then the quadratic contrast was considered in order to determine whether a time- or dose-dependent response was evident. In each case, the data for each sex were analyzed separately. For presentation, standard errors were calculated from the residual mean square in the analysis.

RESULTS

De novo Pheromone Biosynthesis. Both male *I. paraconfusus* and male *I. grandicollis* that had been previously fed on pine phloem (*P. ponderosa* or *P. sylvestris*, respectively) incorporated ^{14}C-acetate into ipsenol, the principal pheromone component for each species (Figure 1A and B). Approximately 170- and 40-times more radioactivity were incorporated into ipsenol by fed *I. paraconfusus* and *I. grandicollis*, respectively, than in comparative acetone-treated controls. Thus, levels of ^{14}C-acetate incorporated into ipsenol in phloem-fed *I. paraconfusus* and *I. grandicollis* were significantly greater than those incorporated into acetone-treated control beetles ($P = 0.014$ and $P < 0.001$, respectively). Consistent with previous work (Seybold et al., 1995b), male *I. paraconfusus* that had been prefed on pine phloem also incorporated ^{14}C-acetate into ipsdienol (Figure 1A), and the ratio of radioactivity in the alcohols was approximately 10:1 (ipsenol:ipsdienol) (see also Figure 2F and legend). The level of ^{14}C-acetate incorporated into ipsdienol in phloem-fed *I. paraconfusus* was significantly greater than that incorporated into ipsdienol in acetone-treated control beetles ($P = 0.023$). Male *I. grandicollis* produced only trace amounts of ipsdienol when fed on *P. sylvestris* phloem, but none when treated with acetone (controls) or JH III. In contrast with a previous study of male *I. pini* (Figure 1C, Tillman et al., 1998), neither *I. paraconfusus* nor *I. grandicollis* that had been previously treated with JH III incorporated notable quantities of ^{14}C-acetate into ipsenol (or ipsdienol in the case of *I. paraconfusus*). The levels of ^{14}C-acetate incorporation into ipsenol in acetone-treated and JH III-treated *I. paraconfusus* and *I. grandicollis* were not significantly different ($P = 0.639$ and $P = 0.580$, respectively). Thus, treatment

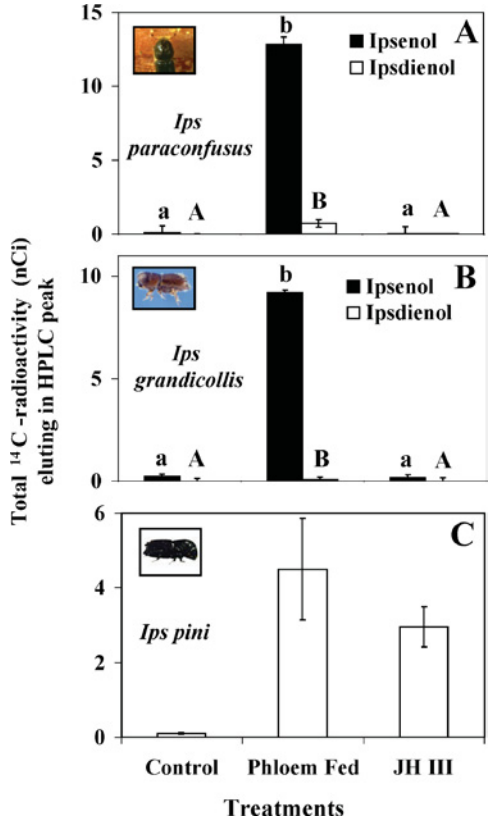

FIG. 1. Effects of feeding on respective host phloem or topical treatment with juvenile hormone III (JH III) on *de novo* ipsenol (■) and ipsdienol (□) biosynthesis in male *Ips paraconfusus* (A), *I. grandicollis* (B), and *I. pini* (C). Studies with *I. paraconfusus* and *I. grandicollis* were conducted in July through September, 2000. Different letters over different histogram bars within a species indicate significant differences in the quantities of radiolabeled ipsenol (lowercase letters) or ipsdienol (uppercase letters). Males were (1) treated topically with 0.5 μl acetone and incubated for 12–16 hr (control); (2) treated topically with 10 μg JH III in 0.5 μl acetone and incubated for 12–16 hr; or (3) fed on *Pinus ponderosa*, *P. sylvestris*, or *P. jeffreyi* phloem, respectively, for 48 hr. Individuals were then injected with $\sim$0.2 μCi sodium [1-^{14}C]acetate, and volatiles were collected from groups of 14 individuals on Porapak Q, while they fed on host phloem for 72 hr. Ipsenol and/or ipsdienol were purified from pentane extracts of the Porapak by HPLC ($N = 4$ groups for each treatment, except for JH III-treated *I. grandicollis* where $N = 3$). Data for *I. pini* (C) are reprinted from *Insect Biochem. Molec. Biol.*, vol. 28, Tillman, J. A, Holbrook, G. L., Dallara, P. L., Schal, C., Wood, D. L, Blomquist, G. J., and Seybold, S. J., *Endocrine regulation of de novo aggregation pheromone production in the pine engraver, Ips pini (Say) (*Coleoptera: Scolytidae), pp. 705–715, 1998, with permission from Elsevier.

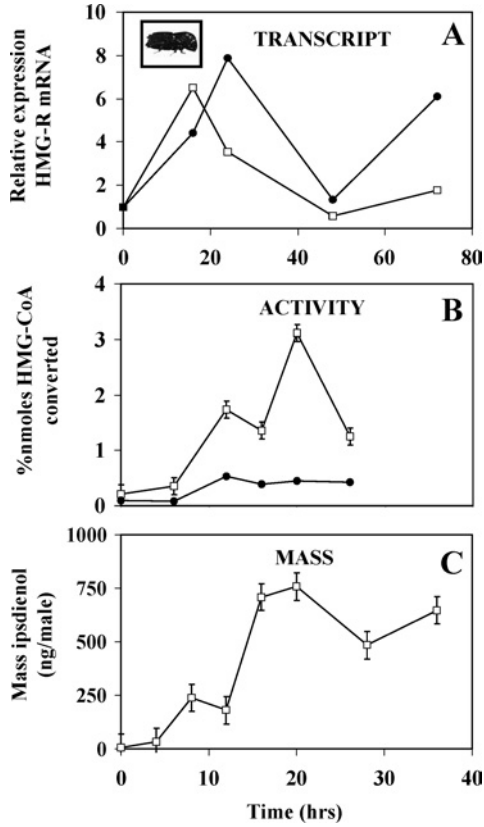

FIG. 2. A–F. HMG-R transcript abundance, *in vitro* HMG-R activity, and accumulation of pheromone in abdominal tissue in male (–□–) and female (–•–) *Ips pini* (A–C) and *I. paraconfusus* (D–F) fed on *Pinus jeffreyi* and *P. ponderosa* host phloem, respectively, for increasing time periods. Means are based on $N = 3$ groups of 8–16 beetles (transcript abundance), $N = 3$ assay samples from one preparation of 40–60 beetles (HMG-R activity), and $N = 6$ groups of 30 beetles (pheromone mass). The mean percentage mass compositions of ipsenol to ipsdienol in *I. paraconfusus* were 80.34 ± 12.81% (4 hr); 81.44 ± 8.33% (8 hr); 82.12 ± 1.80% (12 hr); 82.24 ± 4.84% (16 hr); 82.93±1.64% (20 hr); 82.60 ± 0.19% (28 hr); and 85.34 ± 0.51% (36 hr) (mean ± s.e.). Percentage values ranged from 80.34 to 85.34, with a grand mean over all time points of 82.43 ± 0.58%. Quantities of pheromone alcohols detected at the control condition (0 hr feeding time) were insufficient to calculate a mean value of percentage mass composition. Treatment of recently emerged insects for the studies of HMG-R transcript abundance occurred in May (*I. pini*) and July (*I. paraconfusus)*, 1998; for the studies of HMG-R *in vitro* activity in August (*I. paraconfusus*) and November (*I. pini*), 1998; and for the studies of pheromone accumulation in June and July, 2000 (both species).

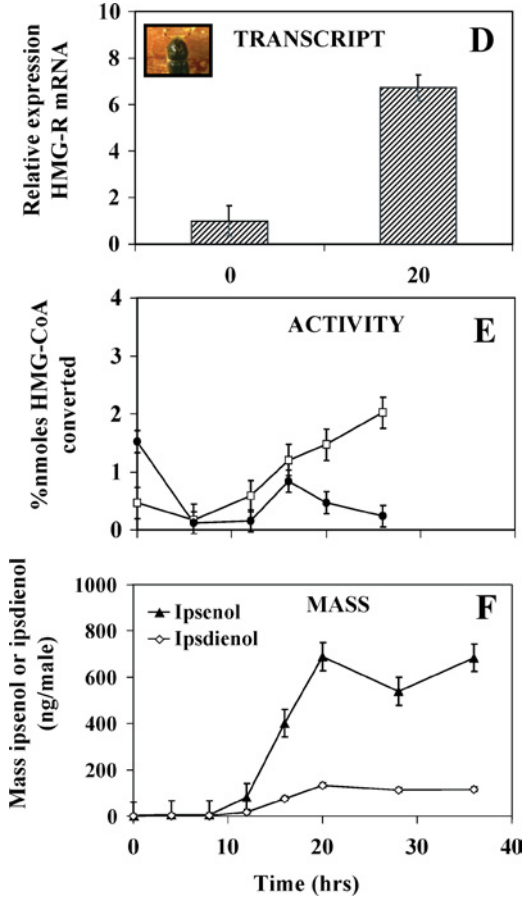

FIG. 2. Continued

with exogenous JH III does not stimulate *de novo* pheromone biosynthesis in *I. paraconfusus* or *I. grandicollis*.

Because this result was different from previous work with *I. pini*, we repeated the experiment with *I. paraconfusus* and *I. grandicollis* and obtained almost identical results (data not shown). The mean masses (±SE) of ipsenol released into the volatile headspace by *I. paraconfusus* during the first experiment were (1) acetone-treated control beetles—233.1 ± 44.5 ng/male ($N = 3$); (2) phloem-fed beetles—501.1 ± 127.8 ng/male ($N = 4$); and (3) JH III-treated beetles—128.3 ± 21.9 ng/male ($N = 4$). Thus, although the mean masses of ipsenol produced by the three treatment groups were of the same order of magnitude, the mean levels of

^{14}C-ipsenol synthesized differed dramatically (Figure 1A). The mass of ipsdienol produced by male *I. paraconfusus* during the first experiment was not quantified, nor were the masses of ipsenol and ipsdienol produced during the second experiment by male *I. paraconfusus* or during either experiment by male *I. grandicollis*.

Accumulation of Pheromone in Abdominal Tissue. Feeding on host phloem stimulated ipsdienol production over time in male *I. pini,* and ipsenol and ipsdienol production over time in male *I. paraconfusus* (Figure 2C and F). In both instances, the studies were conducted during early and mid-summer when pheromone production is not influenced by diapause (Birch, 1974; Lu, 1999). Pheromone production response patterns to feeding on phloem by both species were significant when compared with a linear trend [ipsdienol production by *I. pini* (Figure 2C), linear contrast $F = 90.71$, 1,47 d.f., $P < 0.001$; ipsenol production by *I. paraconfusus* (Figure 2F), linear contrast $F = 131.07$, 1,47 d.f., $P < 0.001$; ipsdienol production by *I. paraconfusus* (Figure 2F), linear contrast $F = 124.57$, 1,47 d.f., $P < 0.001$]. With *I. pini*, detectable levels of ipsdienol were first recorded from males that had fed between 4 and 8 hr in *P. jeffreyi* phloem (Figure 2C), whereas with *I. paraconfusus*, detectable levels of ipsenol and ipsdienol were first recorded from males that had fed between 8 and 12 hr in *P. ponderosa* phloem (Figure 2F). For both species, maximal production was achieved between 16 and 20 hr after feeding had commenced, and remained high through 36 hr at which time the experiment was terminated. The mean maxima for the principal pheromone component for both species were similar (*I. pini*—758 ng/male at 20 hr; *I. paraconfusus*—690 ng/male at 20 hr). At these maxima, production was stimulated about 150 (*I. pini*) and 350 times (*I. paraconfusus*) above the control level measured at 0 hr. In *I. paraconfusus*, the mean percentage mass composition of ipsenol to ipsdienol [100 × (mass ipsenol/{mass ipsenol + mass ipsdienol})] ranged from 80.3% to 85.3% (4–36 hr, respectively, with a grand mean over all times of 82.4%). Taken together with results from the assay for *de novo* biosynthesis (above), the data on pheromone accumulation demonstrate that feeding on phloem stimulates production in both male *I. pini* and *I. paraconfusus*.

The trends in pheromone production with time in *I. pini* and *I. paraconfusus* following treatment with JH III were similar to the trends following feeding (Figure 3C and F inset). In both instances, studies were conducted during the winter (December and January), and may have been under the influence of diapause. However, the pheromone production response patterns by both species at the times following treatment with JH III were significant when compared with a linear trend [ipsdienol production by *I. pini* (Figure 3C), linear contrast $F = 239.61$, 1,47 d.f., $P < 0.001$; ipsenol production by *I. paraconfusus* (Figure 3F inset), linear contrast $F = 30.56$, 1,47 d.f., $P < 0.001$]. In both species, pheromone was first detected between 4 and 8 hr after JH III treatment; although the rise in production was more rapid in *I. pini*. In *I. pini*, the maximum level of production occurred at

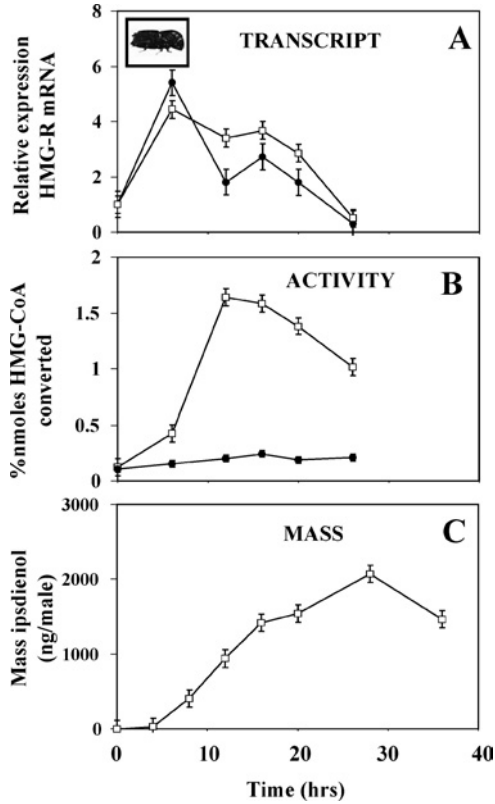

FIG. 3. A–F. HMG-R transcript abundance, *in vitro* HMG-R activity, and accumulation of pheromone in abdominal tissue under increasing incubation time after JH III treatment in male (—□—) and female (—●—) *Ips pini* (A–C) and *Ips paraconfusus* (D–F). Means are based on $N = 3$ groups of 7–15 beetles (transcript abundance), $N = 3$ assay samples from one preparation of 30–60 beetles (HMG-R activity), and $N = 6$ groups of 30 beetles (pheromone mass). Inset in panel F shows attenuated response of pheromone production by male *I. paraconfusus* with time after JH III treatment. Treatment of recently emerged insects for the studies of HMG-R transcript abundance occurred in July (*I. paraconfusus*) and August (*I. pini*), 1998; for the studies of HMG-R *in vitro* activity in October (*I. paraconfusus*) and November (*I. pini*), 1998; and for the studies of pheromone accumulation for both species in December, 1996 and January, 1997.

28 hr (approximately 2000 ng/male), but was uniformly high between 16 and 36 hr. Surprisingly, treatment with JH III during winter stimulated accumulation of ipsdienol in *I. pini* nearly three times more than feeding on phloem did during summer (Figures 2C and 3C). In *I. paraconfusus*, the maximum level of production of ipsenol occurred at 20 hr (approximately 15 ng/male), and remained relatively

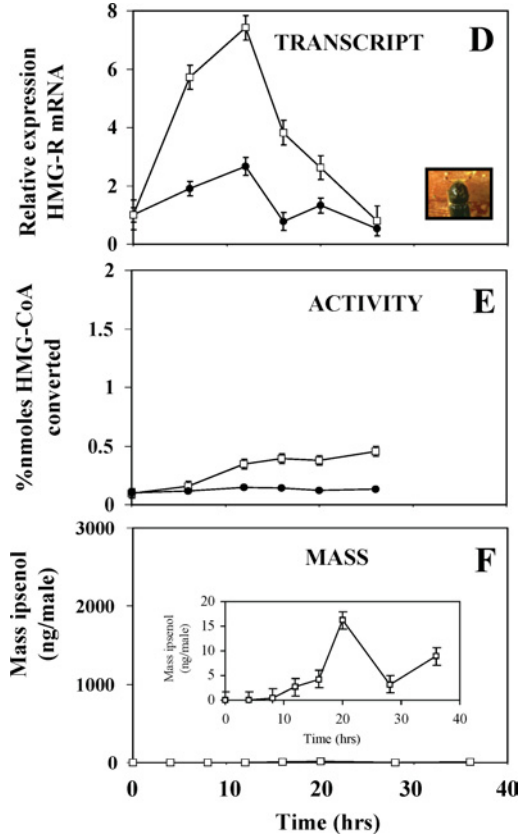

FIG. 3. Continued

high through the end of the experiment (Figure 3F inset). However, the magnitude of maximum ipsenol accumulation in *I. paraconfusus* in response to JH III was about 130 times less than ipsdienol accumulation in *I. pini* (15 vs. 2000 ng/male). Note that the differences in the masses of the principal pheromone components between the two species are evident when responses are viewed on identical scales on the ordinates (Figure 3C and F). In contrast to the situation with *I. pini* and ipsdienol production, treatment with JH III during the winter stimulated about 45 times less ipsenol in male *I. paraconfusus* at 20 hr than feeding on phloem did at 20 hr in the summer (Figures 2F and 3F inset). No ipsdienol was detected in samples from JH III-treated *I. paraconfusus* (Figure 3F inset).

A dose response relationship between JH III and pheromone production by male *I. pini* (Figure 4C) and by male *I. paraconfusus* (Figure 4F inset) was evident. In both instances, studies were conducted during late summer and fall (*I. pini* in

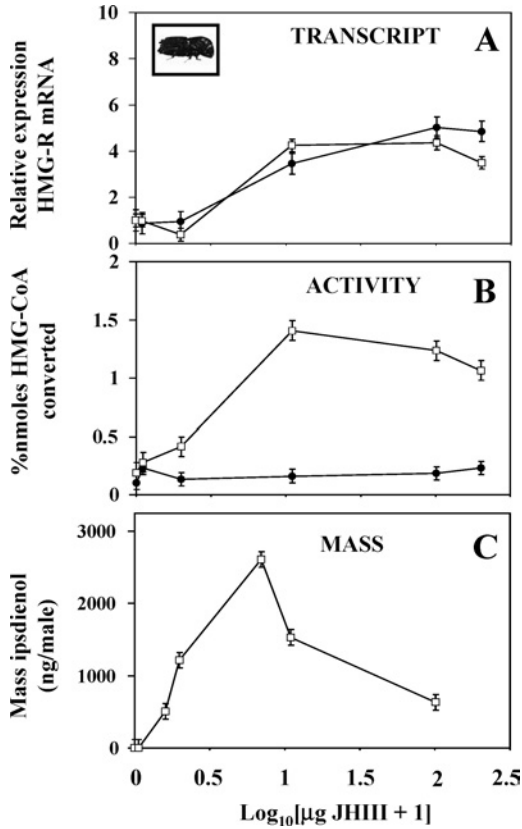

FIG. 4. A–F. HMG-R transcript abundance, *in vitro* HMG-R activity, and accumulation of pheromone in abdominal tissue under increasing JH III dose in male (—□—) and female (—•—) *Ips pini* (A–C) and *Ips paraconfusus* (D–F) following a 20 hr incubation. Means are based on $N = 3$ groups of 7–15 beetles (transcript abundance), $N = 3$ assay samples from one preparation of 30–60 beetles (HMG-R activity), and $N = 12$ groups of 30 beetles (pheromone mass, *I. pini*) and $N = 7$ to 12 groups of 30 beetles [pheromone mass, *I. paraconfusus*—12 groups for all doses of JH III except 1.0 μg (7 groups), 10 μg (11 groups) and 100 μg (10 groups)]. Inset in panel E displays data from a repeated experiment with male *I. paraconfusus* confirming the absence of effect of treatment with JH III on HMG-R activity. Inset in panel F shows attenuated response of pheromone production by male *I. paraconfusus* to JH III dose. Treatment of recently emerged insects for the studies of HMG-R transcript abundance occurred in July (*I. paraconfusus)* and August (*I. pini*), 1998; for the studies of HMG-R *in vitro* activity in July (*I. paraconfusus*) [November, 1998-repeated experiment] and October (*I. pini*), 1998; and for the studies of pheromone accumulation in August (*I. paraconfusus*) and October (*I. pini*), 1996.

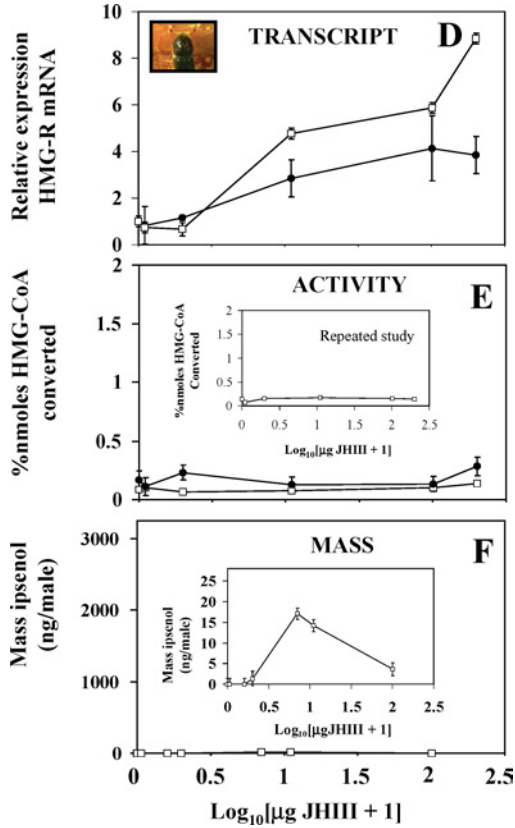

FIG. 4. Continued

October and *I. paraconfusus* in August). Pheromone production response patterns by both species, following treatment with different doses of JH III, were significant when compared with a linear trend [ipsdienol production by *I. pini* (Figure 4C), linear contrast $F = 382.06$, 1,83 d.f., $P < 0.001$; ipsenol production by *I. paraconfusus* (Figure 4F inset), linear contrast $F = 52.91$, 1,75 d.f., $P < 0.001$]. In *I. pini*, ipsdienol was first detected between the doses of 0.06 and 0.6 μg JH III (Figure 4C); whereas in *I. paraconfusus*, ipsenol was first detected between the doses of 0.6 and 1 μg JH III (Figure 4F inset). The rise in production by *I. pini* was much more rapid than in *I. paraconfusus*. In both species, maximal pheromone production occurred at 6 μg JH III/beetle and then tailed off precipitously at the next two higher doses (Figure 4C and F inset). Maximum ipsdienol production by JH III-treated male *I. pini* (approximately 2500 ng/male) again exceeded production following feeding on phloem by 3–4 times (Figures 2C and 4C). Maximum ipsenol

production by JH III-treated male *I. paraconfusus* (approximately 16 ng/male) was again highly attenuated relative to ipsdienol production by phloem-fed male *I. pini* (compare responses on identical scales used on the ordinates of Figure 4C and F), and this level of production of ipsenol by male *I. paraconfusus* in response to JH III was again approximately 45 times less than the level following feeding (Figures 2F and 4F inset). In this case, both experiments were conducted during summer months. No ipsdienol was detected in samples from *I. paraconfusus* treated with various doses of JH III (Figure 4F inset).

In vitro HMG-CoA Reductase Activity.

Feeding on host phloem stimulated HMG-R activity over time in both male *I. pini* (Figure 2B) and male *I. paraconfusus* (Figure 2E). These feeding studies were conducted in November and August in *I. pini* and *I. paraconfusus*, respectively. The HMG-R activity response patterns to feeding on phloem by male and female *I. pini* and male *I. paraconfusus* increased over time when compared with a linear trend [male *I. pini* (Figure 2B), linear contrast $F = 103.26$, 1,17 d.f., $P < 0.001$; female *I. pini* (Figure 2B), linear contrast $F = 121.17$, 1,17 d.f., $P < 0.001$; male *I. paraconfusus* (Figure 2E), linear contrast $F = 31.38$, 1,17 d.f., $P < 0.001$]. With female *I. paraconfusus*, HMG-R activity decreased over time when compared with a linear trend (Figure 2E) (linear contrast $F = 6.67$, 1,17 d.f., $P = 0.021$). With male *I. pini*, a detectable increase in enzyme activity occurred between 0 and 6 hr after feeding commenced, followed by a rather rapid increase between 6 and 12 hr. With male *I. paraconfusus*, the increase did not begin until after 6 hr of feeding, and this was followed by a gradual increase throughout the duration of the experiment. With *I. pini*, maximum activity in the male occurred at 20 hr after feeding commenced (approximately 3% conversion), whereas in *I. paraconfusus* maximum activity occurred at 26 hr after feeding (approximately 2% conversion). In males of both species, the activity curve either coincided with or slightly preceded the mass curve following feeding (Figure 2B,C,E and F). In female *I. pini*, there appeared to be a modest rise in HMG-R activity between 6 and 16 hr after feeding; in female *I. paraconfusus*, there was a modest decline over most time points. Female activity was generally lower than the male activity at all time points (except time 0 hr for *I. paraconfusus*).

The kinetics of HMG-R activity in *I. pini* and *I. paraconfusus* following treatment with JH III were somewhat different from those following feeding (Figure 3B and E vs. Figure 2B and E). These studies with JH III were conducted in November and October in *I. pini* and *I. paraconfusus*, respectively. The HMG-R activity response patterns to JH III treatment by male and female *I. pini* and male *I. paraconfusus* increased over time when compared with a linear trend [male

I. pini (Figure 3B), linear contrast $F = 128.65$, 1,17 d.f., $P < 0.001$; female *I. pini* (Figure 3B), linear contrast $F = 8.20$, 1,17 d.f., $P = 0.014$; male *I. paraconfusus* (Figure 3E), linear contrast $F = 57.73$, 1,17 d.f., $P < 0.001$]. With female *I. paraconfusus*, HMG-R activity was not significant over time following JH III treatment when compared with a linear trend (Figure 3E). As was the case with feeding, treatment with JH III stimulated activity in both species; the induction was abrupt in male *I. pini* and gradual in male *I. paraconfusus*. In *I. pini*, maximal activity occurred sharply between 6 and 12 hr following application with JH III and then declined thereafter. An increase was noticeable even between 0 and 6 hr (Figure 3B). In male *I. paraconfusus*, maximal activity occurred gradually between 20 and 26 hr, with the sharpest increase between 6 and 12 hr after treatment (Figure 3E). In male *I. paraconfusus*, the magnitude of the response of HMG-R activity to JH III over time was attenuated relative to the response in JH III-treated male *I. pini* and attenuated relative to the responses in both phloem-fed male *I. pini* and *I. paraconfusus* (contrast Figure 3E with Figures 3B, 2B, and 2E). In males of both species, the major period of increase in the activity curve in response to JH III treatment preceded the mass curves for ipsdienol and ipsenol, respectively (Figure 3B,C,E and F). Also in both species, male activity curves exceeded female activity curves (Figure 3B and E). As was the case with phloem-fed female *I. pini* (Figure 2B), the activity of the JH III-treated females increased slightly with time (Figure 3B).

The activity of HMG-R increased in response to dose of JH III in male, but not female *I. pini* (Figure 4B) or in either sex of *I. paraconfusus* (Figure 4E). These JH III dose studies were conducted in October and July in *I. pini* and *I. paraconfusus*, respectively. For male *I. pini*, the response to increasing dose of JH III was significant when compared with a linear trend (linear contrast $F = 112.54$, 1,16 d.f., $P < 0.001$). HMG-R activity increased between the doses of 0 and 0.1–1 μg JH III, and then increased sharply between 1 and 10 μg JH III, reaching a maximum at 10 μg before tailing off slightly (Figure 4B). The magnitude of the maximal response of the activity to JH III dose in male *I. pini* (Figure 4B) was not very different from the magnitude of maximal responses with feeding and time (Figure 2B) and with JH III and time (Figure 3B). The dose of JH III that elicited maximal HMG-R activity corresponded to the general dose range (6–10 μg) that elicited maximal ipsdienol production (Figure 4C), and the activity and mass profiles matched fairly well across all doses. Thus, JH III treatment has similar effects on HMG-R activity as does feeding in male *I. pini*. HMG-R activity in female *I. pini*, male *I. paraconfusus*, or female *I. paraconfusus* did not vary with JH III dose. The repeated study with male *I. paraconfusus* gave the same result as the initial study (Figure 4E inset).

FAB-GC-MS Confirmation of HMG-R Activity Assay Product Structure. FAB-GC-MS analyses of both the standard and assay-derived MLL-NE-amide

resulted in a 302 Da product (chromatograms not shown). The molecular weight of MLL-NE-amide is 301 Da, and FAB-GC-MS technique generally produces an ion that is one dalton higher than the actual molecular weight [$(M+H)^+$] of the compound. Thus, this structural evidence supports the assertion that our *in vitro* HMG-R assay is indeed producing mevalonate (via postassay conversion of mevalonate to MLL).

HMG-CoA Reductase Transcript Abundance. HMG-R transcript abundance was higher in male *I. paraconfusus* that had fed on *P. ponderosa* phloem for 20 hr than in unfed males ($F = 44.56$, 1,4 d.f., $P = 0.007$, Figure 2D). A similar short-term increase was observed in male and female *I. pini* (Figure 2A). Transcript levels were approximately 8 and 7 times over background in male *I. pini* (Figure 2A) and *I. paraconfusus* (Figure 2D), respectively, after feeding for similar lengths of time (16 and 20 hr, respectively). Feeding studies were conducted in May and July in *I. pini* and *I. paraconfusus*, respectively.

Topical treatment with JH III resulted in significant relationships between abundance of HMG-R transcript and time after treatment for male and female *I. pini* (Figure 3A), and male *I. paraconfusus* (Figure 3D), but not for female *I. paraconfusus* (Figure 3D). Studies with JH III were conducted in August and July in *I. pini* and *I. paraconfusus*, respectively. For *I. pini*, trends were significant when compared with a linear trend (male *I. pini*, linear contrast $F = 6.81$, 1,17 d.f., $P = 0.023$; female *I. pini*, linear contrast $F = 11.75$, 1,17 d.f., $P = 0.005$). In both cases, after an abrupt increase, the trend was for reduced abundance over time. For male *I. paraconfusus*, the effect of time after JH III treatment on HMG-R transcript abundance was not significant when compared with a linear trend, but it was when compared with a quadratic trend (quadratic contrast $F = 176.03$, 1,12 d.f., $P < 0.001$). The maximal HMG-R transcript levels occurred at 6 hr (for male and female *I. pini*, Figure 3A) and at 12 hr (for male *I. paraconfusus*, Figure 3D) and represented 4.5- and 7.5-fold increases over the background levels at 0 hr, respectively. These maxima in males preceded the times for maximal levels of HMG-R activity and pheromone mass in each species. Female *I. paraconfusus* displayed generally lower HMG-R transcript levels over time relative to males (Figure 3D), whereas female *I. pini* showed levels that were similar to males (Figure 3A). One replicate was omitted from each of the following conditions for *I. paraconfusus* due to insufficient or unsuccessful RNA isolation: female—12 and 16 hr incubation; male—0 and 26 hr incubation.

Topical treatment with JH III resulted in significant relationships between abundance of HMG-R transcript and dose for both sexes of *I. pini* (Figure 4A) and *I. paraconfusus* (Figure 4D). These JH III dose studies were conducted in August and July in *I. pini* and *I. paraconfusus*, respectively. The response patterns increased significantly with dose when compared with a linear trend [male *I. pini* (Figure 4A), linear contrast $F = 113.57$, 1,16 d.f., $P < 0.001$; female

I. pini (Figure 4A), linear contrast $F = 81.02$, 1,17 d.f., $P < 0.001$; male *I. paraconfusus* (Figure 4D), linear contrast $F = 75.11$, 1,16 d.f., $P < 0.001$; and female *I. paraconfusus* (Figure 4D), linear contrast $F = 121.61$, 1,13 d.f., $P < 0.001$]. The dose-response profiles for male *I. pini* and *I. paraconfusus* shared a similar threshold for response (approximately 1 μg of JH III), but the maximum response for male *I. pini* occurred at 10 and 100 μg JH III (approximately four times over background levels at 0 μg JH III), whereas the maximum response for male *I. paraconfusus* occurred at 200 μg JH III (approximately nine times over background). The absence of a decline in response of transcriptional abundance at high doses of JH III for male *I. paraconfusus* was also noted by Tittiger et al. (1999). As was the case with the time course, female *I. paraconfusus* displayed generally lower HMG-R transcript levels over time relative to males (Figure 4D), whereas female *I. pini* showed levels that were similar to males (Figure 4A). Replicates were omitted from each of the following conditions due to insufficient or unsuccessful RNA isolation: male *I. pini*—200 μg; female *I. pini*—0, 1, and 100 μg (two replicates); and male *I. paraconfusus*—1 μg.

DISCUSSION

De novo Pheromone Biosynthesis. The difference in *de novo* pheromone biosynthesis between *I. paraconfusus* and *I. pini* following treatment with JH III is striking. Reasoning that this result may be a true biochemical or molecular difference between *I. paraconfusus* and *I. pini* related to phylogenetic distance and not an experimental artifact, we also repeated the study (twice) with male *I. grandicollis*, a species more closely related to *I. paraconfusus* than to *I. pini* (Wood, 1982). The results suggest a phylogenetic difference in the regulation of pheromone biosynthesis within the genus *Ips*. Other *grandicollis* group species of *Ips* (e.g., *I. lecontei*, *I. montanus*, *I. confusus*, *I. hoppingi,* and *I. cribricollis*) likely regulate their pheromone production in the same manner as the two species tested here. In *I. paraconfusus*, ipsenol is synthesized from ipsdienol (Fish et al., 1979; Vanderwel, 1991). This is presumably the case in *I. grandicollis* as well, but in this species conversion of ipsdienol to ipsenol is nearly complete, with little ipsdienol "byproduct" remaining as a pheromone component. Thus, one hypothetical explanation for the difference in principal component pheromone synthesis between *I. paraconfusus* (or *I. grandicollis*) and *I. pini* is that feeding and topical JH III treatment differentially affect only the last biosynthetic step where ipsdienol is converted into ipsenol. However, our data clearly show (Figure 1A) that the *de novo* biosyntheses of *both* ipsdienol and ipsenol are differentially stimulated by feeding and JH III treatment in *I. paraconfusus*, suggesting that the difference in regulation is associated with earlier reactions in the pathway.

Accumulation of Pheromone in Abdominal Tissue. When feeding on host phloem [*P. jeffreyi* (*I. pini*); *P. ponderosa* (*I. paraconfusus*)] for 20–24 hr, male *I. pini* (ipsdienol) and male *I. paraconfusus* (ipsenol) each accumulate approximately 500–1000 ng/male of the major pheromone component in abdominal tissue (this study). Lu (1999) reported even higher levels of pheromone accumulation following feeding on phloem. Both beetles are able to colonize both pines, and in Porapak aeration studies, both *I. pini* (Seybold et al., 1995a) and *I. paraconfusus* (Seybold, 1992) produce similar quantities of pheromone whether feeding on *P. jeffreyi* or *P. ponderosa*. Thus, host species is not a factor in pheromone production for either beetle species. However, more pheromone is produced during peak spring and summer periods than during fall or winter (Wood and Bushing, 1963; Birch, 1974; Lu, 1999). Background levels of mass in unfed males ranges from 1 to 10 ng/male, so feeding stimulates an approximate 500-fold increase in pheromone production. Both species synthesize their principal pheromone components *de novo* from acetate and mevalonate (Seybold et al., 1995b; Tillman et al., 1998), and phloem feeding stimulates *de novo* ipsdienol and ipsenol biosynthesis in male *I. paraconfusus* (this study) and *de novo* ipsdienol biosynthesis in male *I. pini* (Tillman et al., 1998).

However, in seasonally concurrent, time- and dose-response studies of the relationship between topical application of JH III and mass of principal pheromone components accumulated in abdominal tissue, the maximal levels of production are dramatically different in the two species. At the time that elicits maximal response (*I. pini*, 28 hr; *I. paraconfusus*, 20 hr), JH III induces the accumulation of ipsdienol in male *I. pini* at approximately 130 times the level that it induces accumulation of ipsenol in male *I. paraconfusus* (approximately 2000 ng/male vs. approximately 15 ng/male, respectively). At the dose that elicits maximal response, JH III induces the accumulation of ipsdienol in male *I. pini* at approximately 150 times the level that it induces the accumulation of ipsenol in male *I. paraconfusus* (approximately 2500 ng/male vs. approximately 16 ng/male, respectively). In other respects (JH III levels for threshold, maximum, and high dose inhibition of pheromone mass), the JH III dose–response profiles are similar. The extremely low production of ipsenol by *I. paraconfusus* following topical JH III treatment is consistent with a prior study (Chen et al., 1988) and with our acetate labeling work (Figure 1). Thus, treatment with synthetic JH III alone is not sufficient to stimulate pheromone production to naturally occurring levels in *I. paraconfusus* as it does in *I. pini*.

In vitro HMG-CoA Reductase Transcript Abundance and HMG-CoA Reductase Activity. In male *I. pini*, feeding on host phloem stimulates *de novo* pheromone biosynthesis by inducing the release of JH III from the corpora allata, which then stimulates one or more enzymatically catalyzed reaction(s) (primarily between acetate and mevalonate) in the isoprenoid pathway (Tillman et al., 1998, 1999;

Seybold and Tittiger, 2003). Biosynthetic (Ivarsson et al., 1993; Tillman et al., 1998) and molecular (Tittiger et al., 1999; Keeling et al., 2004) studies with various *Ips* spp. have suggested that HMG-R is one of the enzymes that is regulated during JH-stimulated *de novo* pheromone biosynthesis.

In a replicated and statistically analyzed series of studies comparing *I. pini* and *I. paraconfusus*, we have demonstrated that feeding on phloem and treatment with JH III elicit responses in HMG-R that are commensurate with its involvement in pheromone production (Figures 2–4). The timing of the responses of HMG-R transcript abundance, HMG-R activity, and mass of pheromone accumulated after feeding and exogenous treatment with JH III are all consistent with the roles of JH III and HMG-R in *de novo* pheromone production (Figures 2 and 3). Transcription generally leads activity, which in turn leads accumulation of pheromone.

The relative quantitative changes in HMG-R transcript abundance and activity in response to feeding and exogenous treatment with JH III are also consistent with the roles of JH III and HMG-R in *de novo* pheromone production (Table 3). This relationship is more evident with male *I. pini* than with male *I. paraconfusus*.

TABLE 3. SUMMARY OF RESULTS OF HMG-R TRANSCRIPT, HMG-R ACTIVITY, AND PHEROMONE PRODUCTION IN MALE *Ips pini* AND *I. paraconfusus*: FOLD-INCREASE AT MAXIMUM VALUE RELATIVE TO CONTROL VALUE AND SIGNIFICANCE OF LINEAR OR QUADRATIC TRENDS OF THE RESPONSES[a]

	I. pini		
	Feeding	JH III time	JH III dose
Transcript[b]	7.8[c]	4.5 (SD)	4.3 (S)
Activity[d]	14.3 (S)	13.1 (S)	7.5 (S)
Pheromone (Ipsdienol) production	142.7 (S)	709.2 (S)	2156.3 (S)
	I. paraconfusus		
	Feeding	JH III time	JH III dose
Transcript[b]	6.7[c]	7.4 (SQ)	8.9 (S)
Activity[d]	4.4 (S)	4.7 (S)	1.6 (NS)[e]
Pheromone (Ipsenol) production	341.3 (S)	16.2 (S)	17.1 (S)

[a] S indicates a significant increasing linear trend, SD indicates a significant decreasing linear trend, SQ indicates a significant increasing quadratic trend, and NS indicates no significant trend.

[b] Female trends in transcript: *Ips pini*—JH III time, significant, decreasing linear; JH III dose, significant, increasing linear; *Ips paraconfusus*—JH III time, nonsignificant trend; JH III dose, significant, increasing linear.

[c] No trend analysis was performed.

[d] Female trends in activity: *Ips pini*—Feeding, significant, increasing linear; JH III time, significant, increasing linear; JH III dose, nonsignificant trend; *Ips paraconfusus*— Feeding, significant, decreasing linear; JH III time, nonsignificant trend; JH III dose, nonsignificant trend.

[e] Increase over control for repeated study was 1.3.

Feeding stimulates the level of transcript for HMG-R 7–8-fold in both species. Treatment with JH III induces relative increases in the level of transcript for HMG-R in males of both species that are similar to the increases in fed males (4–9-fold over time and dose). Feeding also stimulates the activity of HMG-R over time (14-fold in *I. pini* and 4-fold in *I. paraconfusus*), and these relative increases are similar to those with JH III over time (13- and 5-fold, respectively). However, despite the relative increase in activity of HMG-R in male *I. paraconfusus* in response to JH III, the activity is low in absolute terms. Further, in two separate experiments, it only increased 1.6- and 1.3-fold with JH III dose, and the trend with increasing dose was not significant (Table 3).

The sex-specificity in HMG-R transcript abundance and activity also reflect the roles of JH III and HMG-R in *de novo* pheromone production by males (Table 3). We have presented data on transcript abundance after normalizing the male results to a male control point and the female results to a female control point, because all male samples were analyzed simultaneously on one gel, whereas all female samples were analyzed within a week on a second gel. Both male and female northern blots were hybridized with the same quantity of the same probe solution. When the data are presented this way, male and female *I. pini* do not appear to differ in the relative changes in HMG-R transcript levels with time or dose (Figures 2A, 3A, and 4A). All trends were significant (Table 3), and trends with time following JH III treatment were significant in a decreasing sense, likely because of the rapid rise in transcript levels between 0 and 6 hr after treatment. In contrast, the amounts of HMG-R transcript in male *I. paraconfusus* relative to the male control points are generally higher than the female amounts normalized to the female control points (Figures 2D, 3D, and 4D). The male trends were significant, but the female trend of JH III treatment with time was not (Table 3). However, a comparison of HMG-R activity in male and female *I. pini* reveals pronounced differences in favor of the males (Figures 2B, 3B, and 4B). Male trends were all significant, but the female trend to increasing doses of JH III was not (Table 3). A comparison of HMG-R activity in male and female *I. paraconfusus* reveals few differences (Figures 2E, 3E, and 4E). Two of the three male trends were significant but the two female trends following JH III treatment were not (Table 3). Thus, activity of HMG-R in females of both species was largely unresponsive to treatment with the hormone over time and dose.

It is surprising that the relative HMG-R transcript levels in *I. pini* did not differ between males and females as dramatically as did the activity levels. In female *I. pini*, the relative increases in transcript abundance with JH III treatment and the weak, but significant increasing trends of HMG-R activity with feeding and JH III treatment over time suggest a role for HMG-R in isoprenoid synthesis in these females. However, it is counterintuitive that transcript abundance should increase nearly identically in a relative sense in males and females, while activity

in males consistently exceeds that in females. This effect may be explained by absolute differences in basal and induced levels of HMG-R transcript. When both the male and female JH III-induced transcript data sets for both species are normalized to the female control points, the male abundances exceed the female abundances by 2–6-fold over time and dose. This semi-quantitative comparison suggests absolute differences in transcript abundance between male and female *I. pini* and *I. paraconfusus* following JH III treatment. Such differences have also been reported recently in phloem-fed male and female *I. pini* where the absolute amount of male HMG-R transcript exceeds the female amount by 10-fold (Keeling et al., 2004).

Quite frequently, our data on HMG-R transcript abundance, HMG-R activity, and pheromone mass suggested a sigmoidal relationship with time or dose. These patterns were not tested statistically because of the low number of data points in each curve. Instead, we tested for the general course of the trends over time or dose using linear contrasts, and in most cases we found that as time or dose increased, the response also increased significantly in comparison to a linear trend. If more data were collected, a regression procedure may have demonstrated the expected sigmoidal trends in our treatments.

Interspecific Comparison. In male *I. pini*, the responses of HMG-R transcript abundance and activity, and pheromone accumulation to JH III dose are all consistent with a major role for HMG-R in *de novo* pheromone production. The comparative dichotomy between JH III stimulation of pheromone production in *I. pini* and *I. paraconfusus* (Figure 1A vs. 1C, 2C vs. 2F, 3C vs. 3F, and 4C vs. 4F) is also supported by the data on HMG-R. Although feeding on phloem stimulates both ipsenol synthesis and HMG-R activity in male *I. paraconfusus*, varying topically applied JH III over time and dose only weakly modulates ipsenol production and HMG-R activity in male *I. paraconfusus*. HMG-R activity in male *I. paraconfusus* increases significantly with time following JH III treatment, but to maximal levels much lower than male *I. pini* or phloem-fed male *I. paraconfusus*; HMG-R activity in male *I. paraconfusus* does not increase significantly with JH III dose. Thus, in male *I. paraconfusus*, the effects of JH III on ipsenol production and HMG-R activity are measurable, but in a highly attenuated sense relative to feeding. With male *I. pini*, treatment with JH III is sufficient to stimulate both ipsdienol production and HMG-R transcript and activity to levels that are similar to the stimulus of feeding on pine phloem. In the case of ipsdienol production in *I. pini*, treatment with JH III elicits a response that even exceeded the feeding treatment. Thus, as was the case with pheromone accumulation, the data on response of HMG-R transcript and activity suggest that synthetic JH III alone is not sufficient to achieve naturally occurring regulation in *I. paraconfusus* as it does in *I. pini*.

Several hypotheses may explain this difference between the two species. First, there could be species-specific differences in the sensitivity to synthetic JH III or to

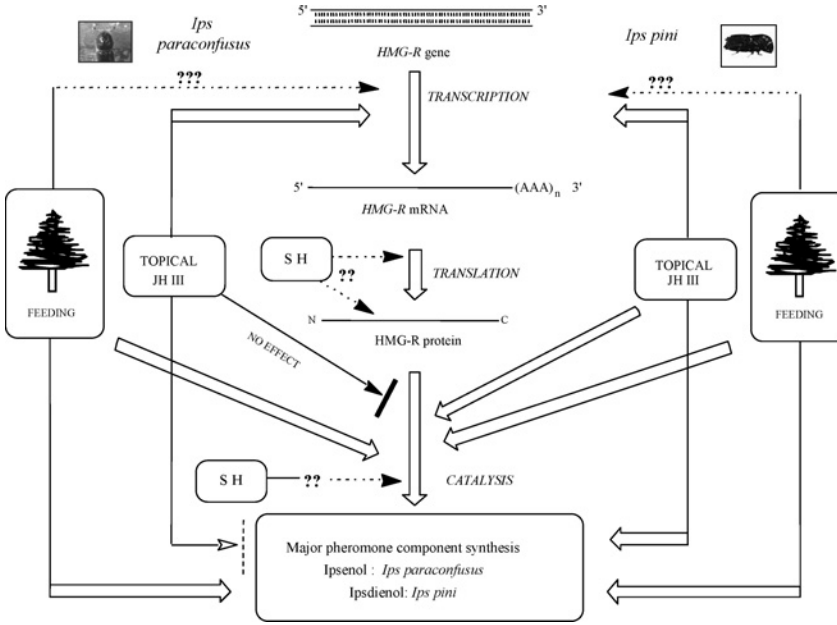

FIG. 5. Model summarizing regulatory aspects of *de novo* aggregation pheromone biosynthesis in *Ips pini* and *I. paraconfusus* (SH = hypothetical second hormone in *I. paraconfusus*).

its enantiomeric composition. However, since JH III does stimulate the abundance of HMG-R transcript in *I. paraconfusus*, this seems unlikely. Secondly, feeding may not stimulate JH III synthesis in *I. paraconfusus* as it does in *I. pini*, but rather feeding stimulates some other hormonal factor involved solely in stimulating *de novo* pheromone biosynthesis. This seems more likely and needs to be tested. Thirdly, perhaps *I. paraconfusus* has a second, feeding-related factor that must interact with JH III in order to stimulate HMG-R activity and ipsenol production (Figure 5). Since the synthesis of ipsenol requires an additional reductive step from ipsdienol in *I. paraconfusus* (Fish et al., 1979; Vanderwel, 1991), it is possible that the second factor may regulate both early- (i.e., HMG-R) and late-stage isoprenoid pathway reactions in *I. paraconfusus*. There is evidence for regulation of a late stage reaction in *I. pini* by JH III (Martin et al., 2003), and evidence for regulation of early- and intermediate-stage reactions in *I. pini* with feeding (Keeling et al., 2004).

The response of HMG-R transcript in male *I. paraconfusus* to increasing doses of JH III is unusual in two aspects. First, as was reported in the unreplicated study by Tittiger et al. (1999), we have confirmed that the level does not decline at even the highest doses of JH III (Figure 4D). The response in male *I. pini*

is more typical of a saturated biological phenomenon (Figure 4A). Second, the transcript in male *I. paraconfusus* is obviously up-regulated with time and dose of JH III (Figures 3D and 4D), but the enzyme that it codes for is not active or much less active than it is in *I. pini* (Figures 3E and 4E). Thus, the postulated second regulatory factor in *I. paraconfusus* appears to be associated with the synthesis, stability, or activity of HMG-R. If this absent posttranscriptional modulatory effect were to be restored in JH III-treated male *I. paraconfusus*, HMG-R activity and pheromone production would conceivably reach levels measured from phloem-fed male *I. paraconfusus*. Since feeding caused a 14-fold increase in HMG-R activity in male *I. pini*, whereas JH III treatment caused 8–13-fold increases, a secondary feeding-associated factor may also be involved to a lesser degree in modulating HMG-R activity in male *I. pini*.

The Potential for Seasonal Effects. Because of the laborious and time-consuming series of replicated studies that we have conducted, we were not physically able to control the season when every aspect of the investigation was carried out. Nonetheless, most of the key experiments (*de novo* pheromone biosynthesis, accumulation of pheromone mass in response to phloem feeding and JH III dose, HMG-R activity assays, and HMG-R transcription assays) were conducted between June and November when these species of *Ips* are presumed not to be in diapause (Birch, 1974). Only the study of pheromone accumulation in response to time after JH III treatment was conducted in the winter months and, in this instance, the male *I. pini* controlled for the potential for diapause by producing large quantities of ipsdienol, whereas male *I. paraconfusus* did not produce large quantities of ipsenol. It is possible that JH III treatment influences pheromone production during periods of diapause differently than does feeding on pine phloem (Birch, 1974). Weighing this evidence we conclude that seasonal effects did not have any bearing on the results of our study.

CONCLUSION

In summary, through quantitative analysis of the mass of pheromone, enzyme assays, and northern analyses, it is clear that *I. pini* and *I. paraconfusus* may regulate *de novo* pheromone biosynthesis differently. These findings lead to a model (Figure 5) for regulation that includes species-specific differences in translational or posttranslational HMG-R regulation by JH III and one or more possible secondary regulatory factors in *I. paraconfusus* and perhaps *I. pini*. A synthesis of these data from both *Ips* spp. indicates that feeding is likely an overarching stimulus for pheromone production, HMG-R activity (through increased translation, enzyme stability, or catalysis), and HMG-R transcript abundance (through increased transcription or delayed transcript degradation). This research on these two closely related bark beetles illustrates the pitfalls of over-generalizing biochemical and

molecular outcomes from one model to all species and emphasizes the benefits of maintaining comparative systems wherever possible.

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INTROGRESSING PHEROMONE QTL BETWEEN SPECIES: TOWARDS AN EVOLUTIONARY UNDERSTANDING OF DIFFERENTIATION IN SEXUAL COMMUNICATION

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Abstract—As a first step toward understanding how noctuid moths evolve species-specific pheromone communication systems, we hybridized and backcrossed two closely related moth species, *Heliothis virescens* (Hv) and *H. subflexa* (Hs), which differ qualitatively and quantitatively in their multicomponent sex pheromone blends. We used amplified fragment length polymorphism (AFLP) marker-based mapping of backcross families to determine which of the 30 autosomes in these moths contained quantitative trait loci (QTL) controlling the percentages of specific chemical components in the pheromone blends. In two previous backcrosses to Hs, we found a strong depressive effect of Hv-chromosome 22 on the percentage of three acetate components in the pheromone gland. These acetates are present in Hs and absent in Hv. Here, we describe how we introgressed Hv-chromosome 22 into the genomic background of Hs. Selection for Hv-chromosome 22 started from backcross 3 (BC_3) females. All females that had Hv-chromosome 22 and a low percentage of acetates (<3% of the total amount of pheromone components present) were backcrossed to Hs males. In BC_5 to BC_8, we determined whether Hv-chromosome 22 was present by a) running only the primer pairs that would yield the markers for that chromosome, and/or b) determining the relative percentages of acetates in the pheromone glands. Either or both genotype and phenotype were used as a criterion to continue to backcross these females to Hs males. In BC_9, we confirmed the isolation of Hv-chromosome 22 in the Hs genomic background, and backcrossed the males to Hs females to eliminate the Hv-sex chromosome as well as mitochondrial DNA. The pheromone composition was determined in BC_3, BC_5, and BC_{11} females with and without

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Hv-chromosome 22. All backcross females with Hv-chromosome 22 contained significantly less acetates than females without this chromosome. In addition, BC_3 females with Hv-chromosome 22 contained significantly more *Z*11-16:OH than BC_3 females without Hv-chromosome 22. However, in BC_5 and BC_{11} females, the correlation between *Z*11-16:OH and Hv-chromosome 22 was lost, suggesting that there are separate QTL for the acetates and for *Z*11-16:OH, and that the relative amount of the alcohol component is only affected in epistasis with other (minor) QTL. Now that we have succeeded in isolating the chromosome that has a major effect on acetate production, we can test in behavioral experiments whether the presence of acetates may have been a driving force for a shift in pheromone composition. Such tests are necessary to move towards an evolutionary understanding of the differentiation in sexual communication in *Heliothis* spp. moths.

Key Words—*Heliothis virescens*, *Heliothis subflexa,* multi-component sex pheromone blend, QTL, quantitative trait loci, backcross, AFLP, amplified fragment length polymorphism, phenotype, genotype.

INTRODUCTION

The number of animal species for which we have even a rudimentary understanding of the genetic control of sexual communication is small, but the few detailed studies involve insect species (Löfstedt, 1990, 1993; Butlin, 1995; Linn and Roelofs, 1995; Phelan, 1997; Coyne and Orr, 1998; Roelofs and Rooney, 2003). Early genetic studies of sexual communication systems focused on determining if the same genes that controlled signal production also controlled signal perception in the opposite sex through pleiotropic effects (sometimes referred to as genetic coupling). Although an early empirical study found evidence supporting the possibility of genetic coupling of acoustic mate communication (Hoy et al., 1977), other studies of both acoustic and chemical sexual communication indicate that such coupling is rare (reviewed in Butlin and Ritchie, 1989). One study that found a genetic correlation between male and female signal and response traits in offspring from field-collected insects determined that these correlations broke down after randomized mating in the laboratory (Gray and Cade, 1999). This indicated that gametic disequilibrium and not pleiotropy (or strong physical gene linkage) had caused the correlation.

Given the lack of evidence for genetic coupling, it becomes difficult to explain the evolution of thousands of moth species, and especially evolutionarily closely related species that have unique pheromone blends. A rare female with a mutation leading to an alteration in her pheromone blend is expected to have lower mating success than normal females, unless males fail to discriminate between the typical and altered blends (Butlin and Trickett, 1997). But in all cases studied, normal males do in fact discriminate against females with atypical pheromonal signals (e.g., Zhu et al., 1997). Similarly, a male with a mutation that results in response

to an altered female pheromone blend is expected to be less efficient at finding typical females. Evidence of this lower efficiency comes from studies of moth genotypes that differ in pheromone responses (e.g., Linn et al., 1997). This selection against new male and female mating traits, when they are at low frequency, is expected to constrain the evolutionary diversification of moth sexual communication systems (Butlin and Ritchie, 1989; Phelan, 1997). Recent efforts to resolve this puzzling situation have focused on understanding if the genetic architecture of variation in signal production and response is structured in a way that would allow coevolution of male and female aspects of sexual signaling in insects (see Phelan, 1997).

So far, the genetic architecture of variation in signal and response has been most thoroughly studied in *Ostrinia* (corn borer) species (Lepidoptera: Pyralidae), which have two-component sex pheromone blends. The ratios of the acetate pheromone components (*Z*)-11-tetradecenyl acetate (*Z*11–14:OAc) and *E*11–14:OAc, produced by females of two races of *O. nubilalis* differ dramatically (97:3 vs. 3:97), and are mostly controlled by a single autosomal gene (Klun, 1975; Löfstedt et al., 1989) that is not linked to a second gene that controls male behavioral response to the pheromone (Löfstedt et al., 1989; Cossé et al., 1995; Linn et al., 1999). Studies of F_2 and backcross progeny from hybridization of other lepidopteran species have also uncovered evidence for single gene control of production of specific pheromone component ratios (Haynes and Hunt, 1990; Jurenka et al., 1994; Zhu et al., 1997). Recently, intriguing data were published indicating that a single change in a desaturase gene caused appearance of a novel pheromone component within the genus *Ostrinia* (Roelofs and Rooney, 2003).

In many related moth species, blend differences involve multiple components, rather than a simple change in the ratio of two components. So far, few studies have focused on the genetic architecture of variation in multi-component blends. A noteworthy exception is a lab-derived *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae) mutant, in which a (single) genetic change in a chain-shortening enzyme was correlated with alteration in multiple component ratios (Haynes and Hunt, 1990; Jurenka et al., 1994; Zhu et al., 1997). Genetic correlations between ratios of multiple components are critical for understanding the evolution of multi-component pheromone blends.

Heliothis virescens (Hv) and *H. subflexa* (Hs) (Lepidoptera: Noctuidae) are two closely related moth species with multi-component pheromone blends. The compounds that are emitted from the pheromone gland of Hv females are *Z*11–16:Ald, 14:Ald, *Z*9–14:Ald, 16:Ald, *Z*7–16:Ald, and *Z*9–16:Ald (Pope et al., 1982; Tumlinson et al., 1982; Teal et al., 1986; Heath et al., 1991). The components that are emitted from the pheromone gland of Hs females are *Z*11–16:Ald, 16:Ald, *Z*7–16:OAc, *Z*9–16:OAc, *Z*11–16:OAc, *Z*9–16:OH, and *Z*11–16:OH (Heath et al., 1991). The behavioral role of some of these components is unequivocal. *Z*11–16:Ald is the most abundant sex pheromone component in both species

and it is essential for attraction in both species. *Z*9–14:Ald is also essential for attraction of Hv males, although this component occurs at only ~5% of the amount of *Z*11–16:Ald in Hv pheromone glands (Roelofs et al., 1974; Tumlinson et al., 1975, 1982; Vetter and Baker, 1983; Ramaswamy et al., 1985; Teal et al., 1986; Vickers and Baker, 1997). Hs males are only attracted when both *Z*9–16:Ald and *Z*11–16:OH are present in addition to *Z*11–16:Ald (Teal et al., 1981; Vickers and Baker, 1997; Vickers 2002). Although these minimal blends attract males, blends comprising all emitted components generally increase male attraction in the field. For example, the Hv minimal two-component blend (*Z*11–16:Ald and *Z*9–14:Ald) caught an average of 7.8 Hv males per night, whereas a seven-component blend (adding 14:Ald, 16:Ald, *Z*7–16:Ald, *Z*9–16:Ald, and *Z*11–16:OH) caught 38.1 Hv males per night (Klun et al., 1980). Several studies tested different combinations of pheromone gland components and found antagonistic effects of some pheromone components. For instance, Hs attraction was reduced when *Z*11–16:OH was present in high amounts relative to *Z*11–16:Ald (Teal et al., 1981; Heath et al., 1990), while Hv male attraction was reduced when the heterospecific component *Z*11–16:OAc was added to the minimal two-component pheromone blend (Vickers and Baker, 1997). Components that have been found in pheromone glands but not in volatile blends (i.e., 14:Ald, *Z*9–14:Ald, and 16:OAc in Hs females, and 14:OH, *Z*9–14:OH, and 16:OH in Hv females) are unlikely to play a role in sexual communication, and their presence is probably a consequence

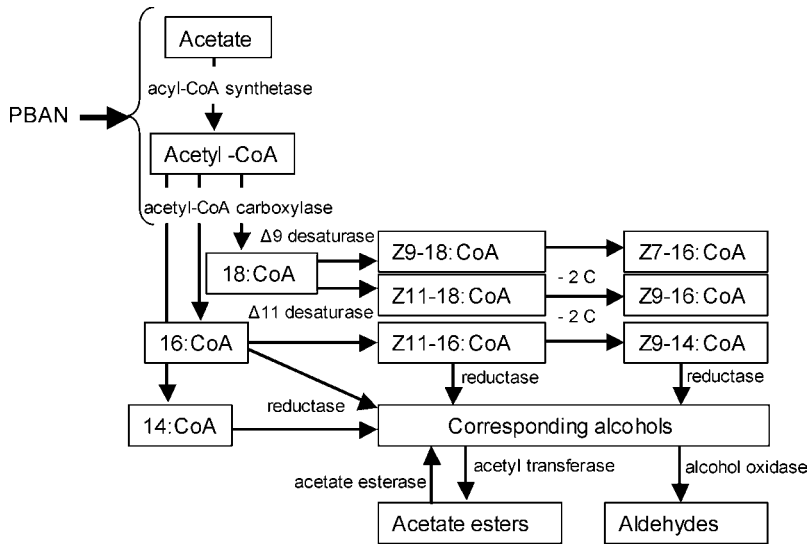

FIG. 1. Generalized biosynthetic pathways of the pheromone components in Hv and Hs. Adapted from Jurenka (2003).

of the biosynthetic pathways of the pheromone components (Figure 1). Because *Z*7–16:OAc and *Z*9–16:OAc have not been tested systematically and independently, their role in attracting Hs males or inhibiting the attraction of Hv males remains unclear.

In a recent study using amplified fragment length polymorphism (AFLP) markers, we found a total of 11 quantitative trait loci (QTL) affecting the amounts of four pheromone components that differ in ratio, or presence/absence, between Hv and Hs (Sheck et al., unpublished). Narrowly defined, a QTL is a single locus that causes a phenotypic effect. However, in practice a QTL is typically defined as a region on a chromosome with little recombination, which contains both a phenotype-altering locus and one or more segments of DNA that can be visualized as markers to indicate the presence of the phenotype-altering locus. In our case, the markers are AFLP fragments, and the region marked is 1 of 31 whole chromosomes because there is no recombination in female Lepidoptera (Robinson, 1971; Turner and Sheppard, 1975; Heckel, 1993; Heckel et al., 1997). In general, Lepidoptera contain 30 autosomes and one sex chromosome, so that each chromosome should, on average, include ~3% of the insect's DNA (Chen and Graves, 1970). The 3% level of QTL resolution is similar to or finer than that in many QTL analyses where recombination is present (e.g., Hawthorne and Via, 2001).

Sheck et al. (unpublished) found the 11 QTL by hybridizing Hv females with Hs males, and subsequently backcrossing F_1 females to Hs males, all in single-pair matings. The pheromone gland contents of BC_1 (first backcross) females were analyzed by GC, and the presence of Hv chromosomes was determined by AFLP markers, after which the amount of phenotypic variation in the relative amount of pheromone components in BC_1 females, that could be explained by presence/absence of one copy of a particular Hv chromosome, was determined. The strongest effects were found for Hv-chromosome 13, explaining 34% of the variance in percentage of 14:Ald, and Hv-chromosome 22, explaining 23% of the variance in percentage of the three acetate esters in the pheromone gland (Sheck et al., unpublished).

Because our long-term goal has been to understand how a single QTL for an alteration in sexual communication can increase in a natural moth population, we wanted to be able to examine the effects of single QTL for sexual communication from one species, when placed in the genomic background of a second, related species. In this paper, we describe how we used a repeated backcrossing procedure to produce hybrid strains that were genetically identical to Hs except for having Hv-chromosome 22. This backcrossing procedure involved a protocol that enabled us to analyze both the pheromone composition and chromosomal make-up of successfully backcrossed, individual moths. We also report data on the pheromone composition of females from these repeated backcrosses that do and do not have a copy of Hv-chromosome 22.

METHODS AND MATERIALS

Moth Strains and Backcross Procedures. In October 2001, 36 single-pair matings were set up, hybridizing *H. virescens* females (YDK strain) with *H. subflexa* males, where each female was identified by using 1–2 letters of the alphabet. The parental strains of Hv and Hs were the same as those described by Sheck et al. (unpublished), and had been in the lab for ~160 and ~40 generations, respectively. Of these 36 single-pair matings, 14 produced at least 30 offspring. Female DD (i.e., the 30th single-pair cross) produced the most offspring, and the female offspring of this cross were backcrossed to Hs males in another set of single-pair matings, to produce BC_1. The 23rd daughter of DD produced the most offspring, henceforth called family DD23, which is the backcross line used for the experiments described in this paper (Figure 2). In all subsequent backcrosses, females from previous backcross families were backcrossed to Hs males in single-pair matings, with the aim of isolating Hv-chromosome 22 in the Hs genomic background.

AFLP Mapping. DNA from individual grandparents, parents, and offspring was extracted, purified, ligated, and amplified, after which AFLP fragments were separated on the basis of size with a LI-COR sequencer (Lincoln, NE). Our AFLP protocol was adapted from Remington et al. (1999) and Vos et al. (1995), and summarized here. We used the Qiagen Qiamp DNA Mini Kit, mousetail protocol with some modifications. DNA was extracted from half of an adult thorax, which was ~20 mg of tissue. Tissue was incubated overnight at 55°C with 180 μl lysis buffer and 20 μl proteinase K per sample, then centrifuged to precipitate the chitin. RNase A (3 μl @ 4 mg/ml) was added to the supernatant and incubated at 37°C for 15 min. The supernatant was adsorbed onto a column, washed with ethanol, and eluted from the column with 70% elution buffer. The final volume was 200 μl per sample. Samples typically had between 2.4–5 μg DNA per 20 mg of tissue. For the restriction step, we started with ≤300 ng of genomic DNA. For a total reaction

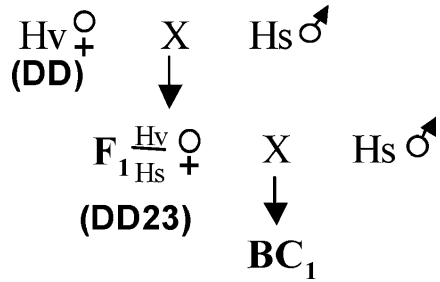

FIG. 2. General mating design for crosses between Hv and Hs, followed by backcrosses to Hs males.

volume of 30.0 μl, we added 6 U of *Eco*R1, 8 U of *Mse*1, and 6 μl of 5 $\times$ R/L buffer. The restriction digest incubated for 2 hr at 37°C, after which we stopped the reaction by incubating at 70°C for 15 min. The ligation step started with 20 μl of restricted DNA. For a total reaction volume of 25 μl, we added 0.5 μl of *Eco*R1 adapter (5 pmol/μl), 0.5 μl of *Mse*1 adapter (50 pmol/μl), 0.5 μl ATP (10 mM), 1 μl 5 $\times$ R/L buffer, 1/2 U of T4 Ligase. This reaction incubated overnight at 37°C or room temperature. The restricted, ligated DNA (R/L DNA) was diluted 1:10. The pre-amplification step was started with 5 μl of the diluted R/L DNA, to which 20.0 μl of preamp mix I (Gibco/BRL), 2.5 μl of 10 $\times$ PCR buffer+Mg (Boerhringer Mannheim), and 2.5 U of *Taq* polymerase were added. We used the following PCR amplification profile: 28 cycles, 30 sec @ 94°C, 30 sec @ 60°C, 60 sec @ 72°C. The pre-amplified DNA was diluted 1:40 by transferring 24 μl of the reaction product to a deep-well plate and adding 936 μl sdH_2O. Selective amplification used 3 μl of pre-amplified R/L DNA. For a total reaction volume of 12 μl, we added 3 μl of M primer (6 ng/μl), 1.2 μl of 10 $\times$ PCR buffer, 0.48 μl of dNTP, 5 mM, 0.14 μl of *Taq* polymerase (5 U/μl), 0.5 μl of IRD labeled E primer (LiCor), and sdH_2O to bring up the volume. The core sequence of the E primer was 5′-GAC TGC GTA CCA ATT C, and the core sequence of the M primer was 5′-GAT GAG TCC TGA GTA A. We added three selective bases to the end of each primer (Sheck et al., unpublished). The PCR amplification profile was as follows: cycles 1–13: 10 sec @ 94°C, 30 sec @ 65°C; −0.7°C/cycle; 60 sec @ 72°C; cycles 14–36: 10 sec @ 94°C; 30 sec @ 56°C; 60 sec @ 72°C+1 sec/cycle. AFLP fragments were separated based on size with a LI-COR 4200 sequencer that, with a scanning laser, simultaneously detects infared labeled DNA fragments of 700 and 800 nm. The samples were prepared for 8% polyacrylamide gels by adding 6 μl of formamide loading dye [95% formamide, 20 mM EDTA, bromophenol blue (USB)] per 12 μl reaction. Samples were denatured at 90°C for 3 min and immediately placed on ice. Samples were loaded into 96 wells (0.7–1.0 μl per well) with a Hamilton syringe. A labeled standard (LI-COR STR marker, 50–700 bp) was loaded at each end of the gel. The original parents and F_1 cross were always loaded into wells two to five. The gels were run for about 3.5 hr, and the images were recorded in a computer file. We scored the gels with a semiautomatic image analysis program designed specifically for AFLP analysis (Quantar 1.08, KeyGene Products).

The markers of interest were those that were present as bands in gels from the Hv parent and the F_1 female, but absent in the original Hs parent or the recurrent Hs backcross parent. For the construction of the DD23 linkage map, we used 88 BC_1 individuals (both males and females), and a total of 14 primer pairs. In order to maximize our chance of finding similar AFLP markers that identify the 31 chromosomes in the different backcross families, we used primer pairs that had previously been used to map the backcross families in which the major effect of chromosome 22 on the production

of acetates was found (i.e., families C5 and C6). In backcrosses subsequent to BC_1, we analyzed females for the presence of Hv-chromosome 22 on the basis of a subset of four AFLP markers that identified Hv-chromosome 22 in DD23.

Pheromone Analysis. Female moths typically cease pheromone production after they have mated. This posed a problem for us, because we needed to determine the pheromone gland content of BC females *after* they had mated and oviposited in order to continue the lines. Therefore, we developed a procedure based on pheromone biosynthesis activating neuropeptide (PBAN) injections that caused mated BC females to produce their normal, genetically determined pheromone blend after ovipositing fertile eggs (Groot et al., 2004). This method was developed subsequent to our finding that the majority of the pheromone glands of BC_1 and BC_2 females had only trace amounts of pheromone compounds after mating and oviposition. The glands of subsequent backcross females were extracted and analyzed as follows (see Groot et al., 2004, for additional details). Females were injected during the photophase with 1 pmol *Hez*-PBAN (Peninsula Laboratories, San Carlos, CA) in 1 μl saline, using a 10 μl syringe with a 31 gage needle (Hamilton, Reno, NV) that was inserted ventrally between the 8th and the 9th abdominal segments. One hour after injection, the pheromone glands were dissected and extracted in conical vials containing 50 μl hexane and 20 ng of 1-pentadecyl acetate (gift from P. Teal) as an internal standard. After 20–60 min, the glands were removed, and the extract was stored at -20°C until analysis. The hexane extracts were reduced to 0.5–1.5 μl under a gentle stream of N_2, and each sample was then injected into a pulsed splitless injector held at 240°C in an HP6890 GC, and separated using a 30 m $\times$ 0.25 mm ID $\times$ 0.5 μm film thickness Stabilwax column (Restek, Bellefonte, PA) temperature programed from 60°C (2 min) to 180°C at 30°C/min, then to 230°C at 2°C/min, during which all the pheromone components eluted. The carrier gas was helium at 30 cm/sec, and the FID was held at 240°C. The amount of each pheromone component was calculated relative to the 20 ng of internal standard. The pheromone components that we distinguished were: 14:Ald, *Z*9–14:Ald, 16:Ald, *Z*7–16:Ald, *Z*9–16:Ald, *Z*11–16:Ald, *Z*7–16:OAc, *Z*9–16:OAc, *Z*11–16:OAc, and *Z*11–16:OH. Most chromatographic analyses did not separate *Z*7–16:Ald from *Z*9–16:Ald. Even though *Z*7–16:Ald is present only in low amounts in both species, and *Z*9–16:Ald is present in relatively large amounts in Hs, we combined the peak areas of these two compounds and denoted the combination as *Z*7/9–16:Ald. The proportions of all pheromone components were calculated as relative percentages of the total amount of pheromone, so that the pheromone composition could be compared among females.

Isolation of Hv-Chromosome 22. After pheromone gland extraction, the backcross females were frozen at -80°C. BC_3, BC_4, and BC_9 females were genotyped with the primer pairs that identified all Hv-chromosomes in DD23 to

determine the presence of any Hv-chromosomes. Selection for Hv-chromosome 22 started with BC_3 females. All females that had Hv-chromosome 22 and a low percentage of acetate esters (i.e., <3% of the total amount of pheromone components present) were backcrossed to Hs males. In BC_5, BC_6, BC_7, and BC_8, we determined whether Hv-chromosome 22 was present a) by running only the primer pairs that would yield the markers for that chromosome and/or b) by determining the relative amount of acetate esters in the pheromone glands. Either genotype or phenotype, or both were used as a criterion to continue to backcross these females to Hs males. In order to completely isolate Hv-chromosome 22, in BC_9 we crossed males to Hs females to eliminate the sex chromosome, as well as mitochondrial DNA, which was inherited from the original Hv female. In the female progeny of this cross, we found that all AFLP markers that identify Hv-chromosome 22 were present, indicating that no recombination had occurred in this chromosome. Backcrosses were only continued with females in which all Hv-chromosome 22 markers were present.

Effect of Hv-Chromosome 22 on Pheromone Composition. In BC_3 to BC_5, and in BC_{11} and BC_{12}, we genetically verified the presence of Hv-chromosome 22, and we analyzed the pheromone composition of all females. ANOVA [PROC GLM in SAS, version 8.02 (SAS Institute, 2002)] was used to assess the effect of presence or absence of Hv-chromosome 22 (i.e., genotype) on the relative amount of the different pheromone components (i.e., phenotype). After BC_9, the only genomic Hv chromosome in any BC female was Hv-chromosome 22. We determined whether the variation in pheromone composition in BC_{11} due to presence or absence of Hv-chromosome 22 was similar to the variation in pheromone composition in BC_3 and BC_5, by comparing the variation of each pheromone component in females with Hv-chromosome 22 between these three backcrosses. The R^2 value from the ANOVA provided an estimate of how much of the phenotypic variation in the production of all pheromone components was explained by the presence or absence of one copy of Hv-chromosome 22.

RESULTS

AFLP Mapping. The primer pairs that we used produced a total of 215 AFLP markers, 193 of which were grouped into 31 linkage groups in the mapping program Mapmaker (version 3, 1993, Whitehead Institute for Biomedical Research). Because there was no recombination, each linkage group represented a whole chromosome. Since we used both males and females, we could easily distinguish the sex chromosome by determining which of the grouped markers were present in females and absent in males. This analysis indicated that linkage group 23 was the sex chromosome. The average number of markers per chromosome was 6.23, with one chromosome (31) containing two markers from

TABLE 1. MEAN AMOUNT OF PHEROMONE IN GLANDS OF MATED, >4-DAY-OLD, BACKCROSS FEMALES INJECTED WITH 1 PMOL *Hez*-PBAN

	BC_3	BC_4	BC_5	BC_6	BC_{10}	BC_{11}	BC_{12}
Amount (ng ± SEM)	293 ± 22	293 ± 19	360 ± 18	270 ± 9	187 ± 22	280 ± 8	250 ± 15
N females analyzed	41	98	147	133	31	205	79
N with <50 ng[a]	1	3	0	3	2	2	3

[a]Females with <50 ng total pheromone.

different primer pairs, one containing three markers, two containing four markers, and the rest containing at least five markers. For chromosome 22, we found one informative marker (223 bp in length, with E-primer AGC and M-primer CTT) that was also present in families C5 and C6 (Sheck et al., unpublished). To confirm the chromosomal homology between families DD23, C5, and C6, we alternated slots on one polyacrylamide gel with C5 individuals having chromosome 22, and C5 individuals lacking chromosome 22, which was repeated for C6 and DD23 individuals. All individuals were chosen based on similarity in presence of other chromosomes, a requirement for finding markers specifically for chromosome 22. Using this procedure we found one other marker that was present in all three groups that had Hv-chromosome 22 (120 bp in length, with E-primer AAC and M-primer CTC). These markers grouped together with eight other DD23-specific markers when using Mapmaker, thus identifying chromosome 22 in DD23.

Pheromone Analysis. Since a number of pheromone components in Hv and Hs are <1% of the total pheromone blend, we found it impossible to accurately determine the relative amount of each component when the gland contained <50 ng of pheromone, as was common in mated females. However, injection of mated, >4-day-old, backcross females with 1 pmol PBAN yielded 187–360 ng of total pheromone in each gland (Table 1). Of the total of 734 BC females from which glands were extracted after PBAN injection, only 14 contained <50 ng pheromone (Table 1). These females were excluded from further analysis.

Isolation of Hv-Chromosome 22. In general, each backcross will result in the loss of (on average) half the chromosomes of the non-recurrent parent, in this case Hv (Figure 3). BC_3 females with Hv-chromosome 22 contained some of the other Hv chromosomes as well (Table 2). However, none of the other Hv chromosomes that were found in these BC females showed a significant effect on the variation in acetate pheromone production (results not shown). Selecting BC_3 females with Hv-chromosome 22 for subsequent backcrossing resulted in 16 out of 29 (55%) of offspring having Hv-chromosome 22 in BC_4 (Table 3). When BC_4 females with Hv-chromosome 22 were backcrossed to Hs males, 39 out of 74 (53%) offspring had Hv-chromosome 22 (Table 3).

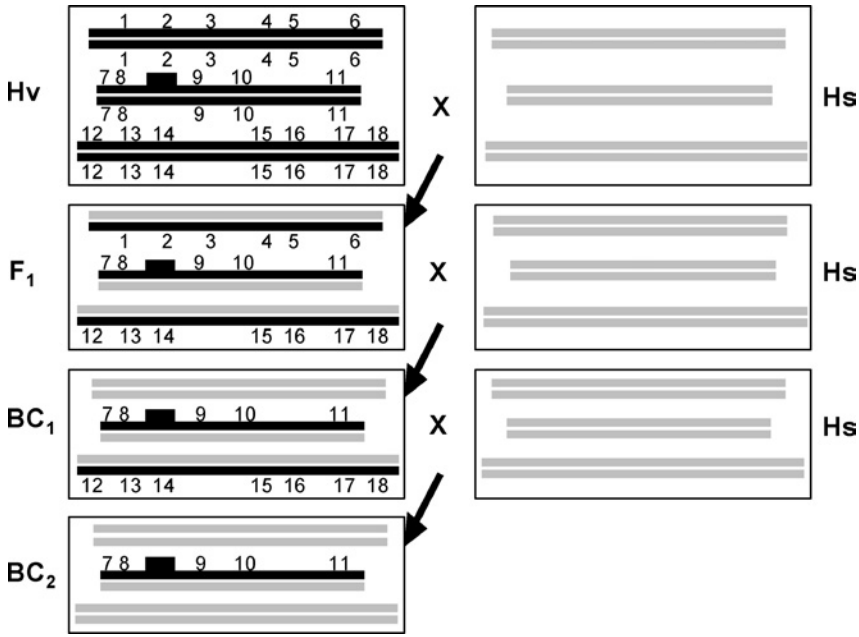

FIG. 3. General schematic example of how recurrent backcrossing with selection (and no recombination in hybrid females) can result in isolation of one Hv chromosome in an Hs genomic background. For simplicity only three pairs of chromosomes, two backcrosses (BC), and one class of the segregating genotypes are shown. Chromosomes from Hv are black, those from Hs are gray. Short black rectangle represents allele coding for pheromone component. Each number represents an AFLP marker mapped to a chromosome.

Backcross females with Hv-chromosome 22 consistently contained a total of $<3\%$ of the three acetate pheromone components in their gland, whereas backcross females without Hv-chromosome 22 typically had $>3\%$ (Figure 4B–D). Hs females and Hv females contained $16.56\% \pm 1.51\%$ (mean $\pm$ SEM) and 0% total acetates, respectively (Figure 4). Therefore, the 3% level was used as a phenotypic threshold for determining the presence or absence of Hv-chromosome 22 up to BC_{12}(Table 4). From BC_{12} on, the total percentage of acetates slightly increased when Hv-chromosome 22 was present, but was never $>5\%$ of the total pheromone content of the gland. In subsequent backcrosses, the percentage of acetates was always contrastingly higher in BC females without Hv-chromosome 22, typically between 10% and 15%, than in BC females with Hv-chromosome 22 (results not shown).

In BC_4 females with Hv-chromosome 22, the total number of Hv-autosomes represented in at least one of the 12 females was reduced to 8 (compared to 19 in

TABLE 2. PRESENCE OF OTHER HV-CHROMOSOMES IN FEMALES FROM THE THIRD, FOURTH, AND NINTH BACKCROSSES

	Number of BC females with Hv-chromosomes				
Hv-chromosome present	In BC_3 with Hv-chromosome 22 ($N = 14$)	In BC_3 all females ($N = 35$)	In BC_4 with Hv-chromosome 22 ($N = 12$)	In BC_4 all females ($N = 42$)	In BC_9 ($N = 7$)
C01	0	0	0	0	0
C02	3	5	5	2	0
C03	1	4	0	0	0
C04	3	7	2	8	0
C05	2	7	0	2	0
C06	0	0	0	0	0
C07	5	7	2	4	0
C08	0	2	0	0	0
C09	0	0	0	0	0
C10	2	4	0	0	0
C11	0	0	0	0	0
C12	1	2	0	2	0
C13	0	0	0	0	0
C14	1	1	0	5	0
C15	0	0	0	0	0
C16	0	0	0	0	0
C17	0	1	0	1	0
C18	5	9	0	0	0
C19	2	4	4	11	0
C20	5	9	2	9	0
C21	3	13	0	0	0
C22	14	14	12	12	7
C23 (sex chr.)	14	35	12	42	3[a]
C24	0	0	0	0	0
C25	6	12	1	7	0
C26	2	4	1	3	0
C27	1	1	0	1	0
C28	2	2	0	5	0
C29	3	7	0	0	0
C30	4	6	0	5	0
C31	6	10	3	8	0

[a] In BC_9, we backcrossed four males to Hs-females to delete the Hv-sex chromosome. The family that retained all Hv-chromosome 22 markers (i.e., family 50-210-46-46-60-712-1716-2625-300) was continued into BC_{10}.

14 BC_3 females) (Table 2). In BC_9 females, the Hv-sex chromosome was the only Hv-chromosome present in addition to chromosome 22. Backcrossing BC_9 males deleted the Hv-sex chromosome, and in one female offspring, all Hv-chromosome 22 markers were retained, indicating that no recombination had occurred in that family. Subsequent backcrosses were conducted with that female.

TABLE 3. INHERITANCE OF HV-CHROMOSOME 22 WITH SELECTION

BC_3-mother[a] with Hv-chromosome 22	BC_4 offspring with Hv-chromosome 22	BC_4 mother* with Hv-chromosome 22	BC_5 offspring with Hv-chromosome 22
4-114-24	3 of 4	4-114-24-63	2 of 3
4-114-34	2 of 3	4-114-34-1	20 of 37
4-114-44	2 of 3		
50-197-9	5 of 9	50-197-9-207	1 of 2
		50-197-7-208	7 of 12
50-210-46	1 of 6	50-210-46-46	1 of 1
50-210-503	3 of 4	50-210-503-203	8 of 19

[a]Name of the mother, designated by a specific number in each generation to keep track of each specific family line.

Effect of Hv-Chromosome 22 in Isolation. In BC_3 females, the percentage of *Z*7-16:OAc was marginally significantly different between females with and females without Hv-chromosome 22 (means ± SEM are 0.43 ± 0.12 and 0.16 ± 0.09, respectively, $P = 0.06$) (Figure 4B). However, the percentages of *Z*9–16:OAc (2.46 ± 0.66 in females without Hv-chromosome 22, and 0.26 ± 0.1 in females with Hv-chromosome 22, $P = 0.006$) and *Z*11–16:OAc (6.94 ± 1.58 in females without Hv-chromosome 22 and 1.28 ± 0.41 in females with Hv-chromosome 22, $P = 0.004$) were significantly different. Also, the cumulative percentage of all acetates was different between BC_3 females without Hv-chromosome 22 and females with Hv-chromosome 22 (9.83 ± 2.31 and 1.69 ± 0.55, respectively, $P = 0.004$). The R^2 value of the latter comparison was 0.20, indicating that 20% of the phenotypic variation in the production of the acetates was explained by the presence/absence of one copy of Hv-chromosome 22.

In BC_5 and BC_{11}, the percentages of all three acetates were different ($P < 0.001$ in both BC's) between females with and without Hv-chromosome 22 (Figure 4C and D). For all three acetates combined, the R^2 value in BC_5 was 0.31, while in BC_{11} it was 0.51. In BC_5 females, *Z*9–14:Ald was also significantly different between females with and without Hv-chromosome 22; females with Hv-chromosome 22 had 0.51% ± 0.06% of Z9-14:Ald, whereas in females without the Hv-chromosome *Z*9–14:Ald represented 0.37% ± 0.04% of the total amount of pheromone ($P = 0.044$, $R^2 = 0.068$). In BC_{11} females, 14:Ald and *Z*11–16:Ald showed differences between the two groups, in addition to the highly significant differences in acetates ($P < 0.001$ for all three acetates). The mean percentage of 14:Ald was 0.35 ± 0.01 in females with, and 0.31 ± 0.01 in females without Hv-chromosome 22 ($P = 0.023$, $R^2 = 0.035$). The mean percentage of *Z*11–16:Ald was 51.1 ± 0.84 in females with, and 47.94±0.84 in females without Hv-chromosome 22 ($P = 0.009$, $R^2 = 0.045$).

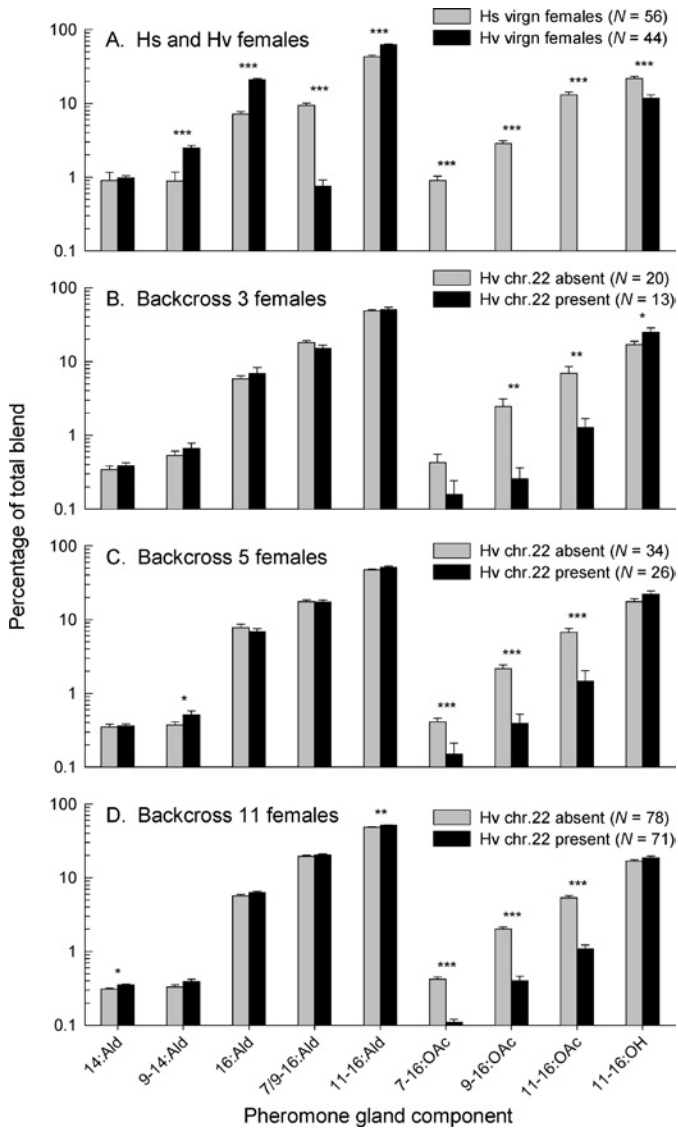

FIG. 4. Pheromone profiles of virgin Hv and Hs females (A), and of BC_3, BC_5, and BC_{11} females (B–D) with and without Hv-chromosome 22 as determined by AFLP analysis. The backcross females were injected with 1 pmol *Hez*-PBAN before glands were extracted. Percentages of pheromone components are graphed on a logarithmic scale to highlight differences between secondary components. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. When no asterisk is shown, differences are not significant.

TABLE 4. ABILITY TO PREDICT GENOTYPE FROM PHENOTYPE

	% Females with >3% acetates (Hv-chromosome 22 absent) or <3% acetates (Hv-chromosome 22 present)[a] (*N*)				
Hv-chromosome 22	BC_3	BC_4	BC_5	BC_{11}	BC_{12}
Absent and >3%	85 (20)	95 (39)	80 (34)	90 (74)	95 (19)
Present and <3%	77 (13)	92 (13)	71 (34)	93 (69)	67 (21)

[a] Percentages of females with Hv-chromosome 22 absent that had a total percentage of acetates >3% of the total pheromone blend, and females with Hv-chromosome 22 present in which the total percentage of acetates was <3% of the total pheromone blend.

DISCUSSION

By using phenotypic as well as genotypic (marker-specific) traits, we have successfully isolated Hv-chromosome 22 in an Hs nuclear and mitochondrial genomic background. Although Hv females do not contain any acetate esters in their pheromone gland, BC females with a single copy of Hv-chromosome 22 generally contained a small amount of acetate components, which usually summed to <3% of the total pheromone blend. The fact that acetate production is not completely shut down in females with Hv-chromosome 22 suggests that the heterozygous presence of this chromosome is not enough to completely inhibit acetate accumulation in the glands. The absence of acetates in Hv female glands could be due to more than one specific step in the biosynthetic pathway (Bjostad and Roelofs, 1983; Morse and Meighen, 1986; Teal and Tumlinson, 1987). In general, the acetates are thought to be synthesized from the corresponding alcohols through acetyl transferase (Figure 1), and a difference between Hv and Hs in presence or activity of this enzyme could explain the different amounts of acetate esters in their respective pheromone blends. However, acetate esters can be converted back to the corresponding alcohols through acetate esterase (Figure 1). Teal et al. (1989) found significantly higher acetate esterase activity in Hv pheromone glands, suggesting that any acetate esters produced by Hv would be immediately converted back to the corresponding alcohols. Thus, an alternative explanation for the differences between Hv and Hs might involve the presence or activity of acetate esterase.

In BC_3 females, the percentage of *Z*11–16:OH significantly correlated with presence of Hv-chromosome 22, in addition to the percentages of the acetate esters. A similar result has been found in the previous C5 and C6 BC_1 females, and matches with the negative phenotypic correlation between *Z*11–16:OH and each of the three acetates (Sheck et al., unpublished). It could be that the factor associated with chromosome 22 contributes to conversion of acetates to alcohols (acetate esterase). Alternatively, there may be separate QTL on chromosome 22 that control acetate and alcohol production. In BC_5 and BC_{11} females, the correlation between *Z*11–16:OH and Hv-chromosome 22 was lost. This suggests that presence of

Hv-chromosome 22 alone in an Hs genetic background is not sufficient to affect *Z*11–16:OH levels. It may be that QTL on Hv-chromosome 22 interacts epistatically with QTL on other Hv chromosomes to affect the level of this alcohol.

In BC_5 females, *Z*9–14:Ald was significantly correlated with the presence of Hv-chromosome 22, while in BC_{11} females, 14:Ald and *Z*11-16:Ald showed significant differences between females with and females without Hv-chromosome 22. However, the statistically significant differences in the relative amounts of 14:Ald and *Z*9–14:Ald may not be biologically significant, because the differences in the quantities produced were very small. Previously, we confirmed the presence of small amounts of these components in Hs females (Groot et al., 2004). The percentage of 14:Ald relative to the main component was similar in virgin Hs and virgin Hv females (Figure 4A), suggesting that the significant differences in BC_{11} may indeed be an artifact due to the low amount of 14:Ald. The observation that the major component, *Z*11–16:Ald, was significantly higher in BC_{11} females with than in females without Hv-chromosome 22, is likely due to the interdependence of the different components. Since all other components are similar between the two groups, and females without Hv-chromosome 22 contain significantly higher amounts of acetates, the percentage of the major component is likely to be lower in those females based on carbon flow in the biosynthetic pathway (Figure 1). The R^2 value was very low, only 4.5% of the phenotypic variation in the production of Z11–16:Ald was explained by the presence/absence of one copy of Hv-chromosome 22, while 51% of the phenotypic variation in the production of the acetates was explained by the presence/absence of this chromosome.

The effect of Hv-chromosome 22 on the variation in pheromone composition is only the first step toward our long-term goal of understanding the evolutionary processes that have resulted in diversification of moth sexual communication signals and responses. Currently, we are conducting cage and field experiments with the introgressed lines described here to determine how the difference in this chromosome, and the corresponding reduction of acetate production, affect the likelihood of a female being mated by an Hv or Hs male. So far, acetates have not been rigorously demonstrated to function as attractants for Hs males (Teal et al., 1981; Tumlinson et al., 1982; Vickers, 2002), but Vickers and Baker (1997) have shown that *Z*11–16:OAc deter Hv males. The simplest evolutionary scenario for evolution of presence of acetates would be that deterring heterospecific males would have been a driving force for a shift in the pheromone blend to which acetates were added. Now that we have succeeded in isolating the chromosome that has a major effect on acetate production, we can test this hypothesis through behavioral experiments. Such tests are necessary to move towards an evolutionary understanding of the differentiation in sexual communication in *Heliothis* spp. moths with multi-component sex pheromone blends.

Fine-scale mapping and cloning of the QTL can help explain which gene(s) are involved in control of acetate production, and how they interact biochemically.

Currently, we are saturating the chromosome with as many AFLP markers as we can (we now have 18 markers; J. L. Bennett, unpublished results), and we are conducting backcrosses in which the Hs parent is the female and the male parent is from the introgression line carrying the Hv-chromosome 22. Because there is crossing-over in male chromosomes, this type of cross allows recombination between segments of Hv- and Hs-chromosome 22. The offspring of these crosses will be used for developing lines that are homozygous for only a small segment of Hv-chromosome 22 that contains the QTL for acetate levels. Also, specific differences in the biochemical pathways of the pheromone components between Hv and Hs are currently being unraveled (Choi et al., 2004), which can lead to the identification of candidate genes. Earlier we have demonstrated that it was possible to use a combination of mapping and candidate genes to identify and sequence one important gene in Hv (Gahan et al., 2001). With the identification of genes underlying pheromonal differences between the species, the sequences of these genes in different geographic populations can be examined to determine patterns of synonymous and non-synonymous substitutions within and between species, indicating the action of selection (e.g., Taylor et al., 1996; Tsaur et al., 1998). The patterns of allelic substitution and diversity in these species will provide insights about which substitutions were critical in the diversification of the signal/response system (e.g., Saez et al., 2003).

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EFFECT OF HOST DEFENSE CHEMICALS ON CLONAL DISTRIBUTION AND PERFORMANCE OF DIFFERENT GENOTYPES OF THE CEREAL APHID *Sitobion avenae*

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Abstract—Five microsatellite loci were used to study the genetic variability and population structure of *Sitobion avenae* (Hemiptera: Aphididae) on some of its host plants. Individuals were collected in Chile from different cultivated and wild Poaceae. Forty-four multilocus genotypes were found among the 1052 aphids analyzed, of which four represented nearly 90% of the sample. No specialist genotypes were found, although some preferred hosts endowed with chemical defenses, *i.e.*, hydroxamic acids (Hx), while others preferred comparatively undefended hosts. Performances of some predominant and some rare genotypes were evaluated on plants differing in their Hx levels. Significant differences in performance were found among clones, the two most common genotypes showing no differences in performance among all hosts tested, and the rare genotypes showing enhanced performance on the host with highest Hx level. A hypothesis is proposed whereby the appearance of rarer genotypes is in part related to the presence of Hx.

Key Words—*Sitobion avenae*, microsatellites, genetic diversity, host structuring, chemical defenses, PCR analysis.

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INTRODUCTION

Host secondary chemistry constitutes one of the main factors guiding the evolution of plant–insect interactions (Scriber, 2002; Becerra, 2003), via the selection of phytophagous insect populations (Erlhich and Raven, 1964). Aphids, which are mostly found as clonal populations, are highly host-specific insects (Dixon, 1998); hence, genetic differentiation according to the host plant and the existence of biotypes or host races specifically adapted to some host species may occur (Via, 1991; Vanlerberghe-Masutti and Chavigny, 1998; Lushai et al., 2002; Massonnet et al., 2002; Via and Hawthorne, 2002; Miller et al., 2003; Simon et al., 2003). In cereal aphids, the host plant has a strong impact on genetic structure and clonal diversity of populations, as evidenced by several studies using molecular markers (De Barro et al., 1995a,b; Simon and Hebert, 1995; Lushai et al., 1998; Haack et al., 2000; Figueroa et al., 2002). In particular, temporal and spatial clonal structures based on different host plants have been demonstrated in *Sitobion avenae* (Fabricius) (Caillaud et al., 1995; De Barro et al., 1995a,b; Figueroa et al., 2002). However, no attempt has been made to relate host-based population structure with the occurrence of specific secondary metabolites in the plant.

Hydroxamic acids (Hx) are the main group of secondary metabolites involved in cereal resistance against aphids (Niemeyer, 1988; Niemeyer and Pérez, 1995; Sicker and Schultz, 2002). Hx exist in the intact plant as glucosides (Cambier et al., 1999), which are hydrolyzed to the more toxic aglucones by endo-β-glucosidases when the tissue is injured (Hofman and Hofmanova, 1969; Cuevas et al., 1992; Sue et al., 2000) (Figure 1). DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one), the main Hx aglucone in wheat extracts (Sicker et al., 2000), produces antibiosis, feeding deterrence, and decreased performance and reproduction in aphids (Niemeyer and Pérez, 1995), and exhibits mutagenic effects (Hashimoto et al., 1979; Hashimoto and Shudo, 1996). More recently, it has been shown to affect the level of genetic polymorphism in aphid populations (Figueroa et al., 2002).

Sitobion avenae was introduced in Chile in the 1970's (Apablaza, 1974), and has invaded both cultivated and wild Poaceae, such as wheat, oat, barley, maize, cocksfoot grass, and wild *Hordeum*. In order to test whether the population genetic

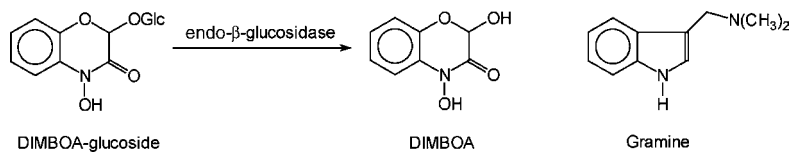

FIG. 1. Secondary metabolites in cereals.

structure of an aphid species is affected by the chemistry of its host plants, the genetic diversity and clonal distribution of *S. avenae* in Chile according to host plants differing in their chemical defenses was examined. It was hypothesized that if a differential distribution of genotypes of *S. avenae* on hosts occurs, this could be a consequence of different adaptive responses of aphid genotypes to plant chemistry. To test this hypothesis, the performance of different *S. avenae* genotypes on hosts differing in Hx level was evaluated.

METHODS AND MATERIALS

Aphid Sampling. *Sitobion* aphids were sampled in Central–South Chile (from 33° to 41°S lat.) on available hosts where they were present at the time and site of collections. The survey included crops of durum wheat (*Triticum durum* L.) and oat (*Avena sativa* L.), and field margins and other surrounding areas with cocksfoot grass (*Dactylis glomerata* L.), wild oat (*Avena fatua* L.), and mouse barley (*Hordeum murinum* L.). In fields, the collection was performed throughout a linear transect, irrespective of the level of aphid infestation and host plant abundance. On field margins, the collection depended on the distribution of the plants. In both cases, an individual aphid was collected from a plant separated by at least 10 m from the last sample in order to limit the chance of resampling individuals from the same parthenogenetic mother. Samples were collected in 95% ethanol and preserved at −20°C prior to their utilization. *Sitobion* individuals were determined as *S. avenae* or *S. fragariae* according to Figueroa et al. (1999b).

DNA Extraction and PCR Amplifications. Genomic DNA was extracted from single wingless adult aphids according to Sunnucks and Hales (1996), and the DNA was precipitated in ethanol and resuspended in 20–40 μl of sterile ultrapure water depending on the aphid size. PCR amplifications of microsatellite loci (*Sm10, Sm11, Sm17, S3.R*, and *S5.L*; Sunnucks et al., 1996; Simon et al., 1999; Wilson et al., 2004) were prepared in a 15 μl reaction volume, including 0.5 units of *Taq* DNA polymerase (Invitrogen, USA), Mg^{2+}-free reaction buffer, 2 mM $MgCl_2$, 200 μM dNTPs, 10 pmol of each primer (BiosChile-IGSA, Chile), and about 10 ng of aphid DNA. PCR reactions were carried out in a Perkin-Elmer 9700 thermocycler using the following steps: an initial denaturation for 2 min at 94°C, and 40 cycles consisting of denaturation for 40 sec at 94°C, annealing for 45 sec with temperature depending on locus (Sunnucks et al., 1996; Simon et al., 1999), and elongation at 72°C for 45 sec. For the last cycle, the elongation time was extended to 4 min. The PCR reaction was mixed with 4x loading buffer (Sambrook et al., 1989), denatured for 3 min at 95°C, loaded on to a 6% polyacrylamide-urea gel, and subjected to electrophoresis in 0.5X TBE buffer at 1.0 kV. After electrophoresis, the gel was silver stained as described in Haack et al. (2000). The

size of the alleles of each locus was estimated by using a sequencing size ladder corresponding to the sequence of pGEM®-3Zf(+) vector (Promega, USA).

Genetic Analysis. Allelic polymorphism was examined at five microsatellite loci. Each multilocus genotype was characterized by its unique allelic combination at the five loci. Each host plant was considered as a population, and all subsequent analyses were performed on this sampling unit. The effect of the host plant on the distribution of genotypes was analyzed using Fisher's exact tests (Fisher, 1925).

Performance of Aphid Genotypes. Monoclonal colonies, which started from individual field-collected aphids, were maintained on oat for at least three generations. Seven genotypes were selected according to their prevalence and their host range: four very frequent and widely distributed genotypes (Sa1, Sa2, Sa3, and Sa4; Table 1 and Table 2), two genotypes with a few collections (Sa7: 14 copies; and Sa10: 5 copies), and one single-collection-genotype (Sa36). The rare genotype Sa7 was detected on oat ($N = 8$), cocksfoot grass ($N = 2$), and wheat ($N = 4$), whereas Sa10 was restricted to oat ($N = 1$) and wheat ($N = 4$). The unique genotype Sa36 was only detected on wheat. The performance of each of these genotypes was evaluated on host plants with different levels of Hx, i.e., oat (no Hx; control), and wheat cultivars differing in their Hx concentration: low Hx cv. Huayún and high Hx cv. Chagual (mean ± SE: 0.667 ± 0.097 and 3.595 ± 0.257 mmol/kg fresh wt, respectively, $N = 10$, two-leaf seedlings). Although these wheat lines are not isogenic, negative correlations between Hx level and aphid performance have been described, which include intra- and interspecific variation in cereal taxa (e.g., Argandoña et al., 1980). From each genotype (clone), one 5-day-old larva (synchronization of larvae to *ca*. 12 h) was transferred to a potted 8-day-old seedling and enclosed in a clip-cage. Larvae were observed daily, and the pre-reproductive period was recorded (T). The progeny produced in the subsequent T days was counted (Md) and removed daily. The intrinsic rate of increase (r_m) (Birch, 1948), a synthetic estimate of performance, was determined by the equation of Wyatt and

TABLE 1. SAMPLE SIZE, NUMBER OF GENOTYPES, AND FREQUENCY OF THE FOUR MOST FREQUENTLY OCCURRING MULTILOCUS GENOTYPES OF *Sitobion avenae* PER HOST PLANT

	Host plant					
	Oat	Wild-oat	Mouse barley	Cocksfoot grass	Wheat	Total
Sample size	175	27	18	70	762	1052
Number of genotypes	15	4	4	15	35	44
Sa1 (378 collections)	0.12	0.04	0.39	0.04	0.45	0.36
Sa2 (340 collections)	0.46	0.37	0.28	0.54	0.27	0.32
Sa3 (127 collections)	0.13	0.33	0.28	0.01	0.12	0.12
Sa4 (75 collections)	0.17	0.26	0	0.13	0.04	0.07
Sa5–Sa44 (132 collections)	0.12	0	0.05	0.28	0.12	0.13
Total	1.00	1.00	1.00	1.00	1.00	1.00

TABLE 2. SITES WHERE DIFFERENT CLONES OF *Sitobion avenae* WERE COLLECTED

Zone	Main focal cities	Mean latitude	Clones collected
1	Santiago-Rancagua	33° S	1-2-3-4-6-7-14-15-16-17-20-26-27-34-37-42-43
2	Talca	35° S	1-2-3-4-6-7-14-21-23-24-32-38-41
3	Chillán-Los Angeles	37° S	1-2-3-4-7-8-10-35
4	Temuco	39° S	1-2-3-4-5-7-8-9-10-11-12-13-16-18-19-20-22-25-28-29-30-31-33-39-40-44
5	Osorno	41° S	1-2-3-36

White (1977): $r_m = 0.738$ (ln Md)/T. A range of 7–16 replicates was used per genotype. Statistical significance for all performance comparisons was computed by using two-way ANOVA (factors: aphid genotype and host) with the STATISTICA package (StatSoft, 2004); multiple comparisons were performed with a LSD test in the same program. Performance data were adjusted to normal distribution by using the logarithmic transformation (Sokal and Rohlf, 1981).

RESULTS

Genetic Diversity and Structuring of Sitobion avenae *Populations.* Combining the five microsatellite loci, 44 multilocus genotypes were characterized among the 1052 sampled individuals of *S. avenae*. As a result of this clonal amplification, genotypic diversity was very low on each host plant as well as in the complete data set (Table 1). Overall, only 4% of the collections of Chilean *S. avenae* consisted of unique genotypes while the four most abundant genotypes (Sa1–Sa4, Table 1 and Table 2) represented nearly 90% of the sample. This pattern suggests that populations of *S. avenae* in Chile are mainly determined by clonal reproduction of a few genotypes, which is confirmed by strong deviations from Hardy–Weinberg equilibrium and frequent linkage disequilibrium in these populations (data not shown). Since each five-locus genotype should represent a clone, the population structure was analyzed in terms of clonal frequencies, thus studying the effects of host plants on the distribution of the clones.

Distribution of Sitobion avenae *Genotypes.* The frequency of all multilocus genotypes was compared among the five host plants. The global effect of hosts on frequency distribution of genotypes was significant (Fisher's exact test, $P < 0.01$). Differences were observed among the genotypic frequencies in most host plant comparisons (Fisher's exact test, $P < 0.05$). When only the three most-infested host plants (wheat, oat, and cocksfoot grass) were considered, differences were observed between oat and cocksfoot grass ($P < 0.03$), and highly significant differences were observed between aphid populations on wheat and cocksfoot grass ($P < 0.001$), and on wheat and oat ($P < 0.001$). Interestingly, these differences

were observed between host plants with chemical defenses against aphids (wheat) and host plants without such defenses (oat and cocksfoot).

Since some rare (less than 22 individuals collected; Sa5–Sa22) or unique genotypes (Sa23–Sa44) of *S. avenae* were restricted to a single host plant (20 genotypes on wheat, seven on cocksfoot grass, and two on oat), an additional comparison was performed that included the four more frequent *S. avenae* genotypes (Sa1–Sa4). These most frequently occurring genotypes contributed to more than 80% of the total variance, the remaining 20% of the variation being explained by the bulk of rare genotypes (Sa5–Sa44). Hence, subsequent analyses were performed considering only the four most frequent genotypes (Table 1). Significant differences were found between genotypic frequencies when they were sampled from all five hosts ($P < 0.001$), and when they were sampled only from wheat, oat, and cocksfoot grass ($P < 0.001$).

Effect of the Host Plant on Performance Sitobion avenae *of* Genotypes. Performances, as measured by r_m, showed highly significant effects for the genotype (two-way ANOVA's, $P < 0.002$), the host plant ($P < 0.02$), and their interaction ($P < 0.03$, Figure 2). At an inter-host level, the frequent and widely distributed genotypes, Sa1 and Sa2, exhibited comparable performances on the three hosts tested ($P > 0.05$ on three possible host comparisons), while the remaining genotypes exhibited a higher performance on the host with highest Hx level. At an intra-host level, differences between clones were observed only when Hx were present in the host. Thus, on cv. Chagual, two groups of genotypes showed highly significant differences among their performances: genotypes Sa3, Sa4, Sa7, Sa10, and Sa36 exhibited a significantly higher performance than Sa1 and Sa2 (*a posteriori* test, $P < 0.004$), while on cv. Huayún, genotypes Sa4 and Sa10 showed a higher performance ($P < 0.03$), but only in comparison to genotype Sa2. Hence, Hx are involved in the effect of host and aphid genotype on performance, and the effect of the interaction between host and aphid genotype may also be related to the levels of Hx. This effect on performance is predominately observed for rare and unique aphid genotypes, which are mainly detected on wheat in the field.

DISCUSSION

The distribution of multilocus genotypes characterized in Chilean populations of *S. avenae* appears to be influenced by the presence of chemical defenses in their host plants. No specialized genotypes (i.e., collected on a single host plant) were found. However, some genotypes seem to be more specialized than others, as shown by their different collection frequencies on the various plant species examined (Table 1). For example, the second most frequently occurring genotype, Sa2, occurred at similar frequencies on all host plants, while genotype Sa1 showed

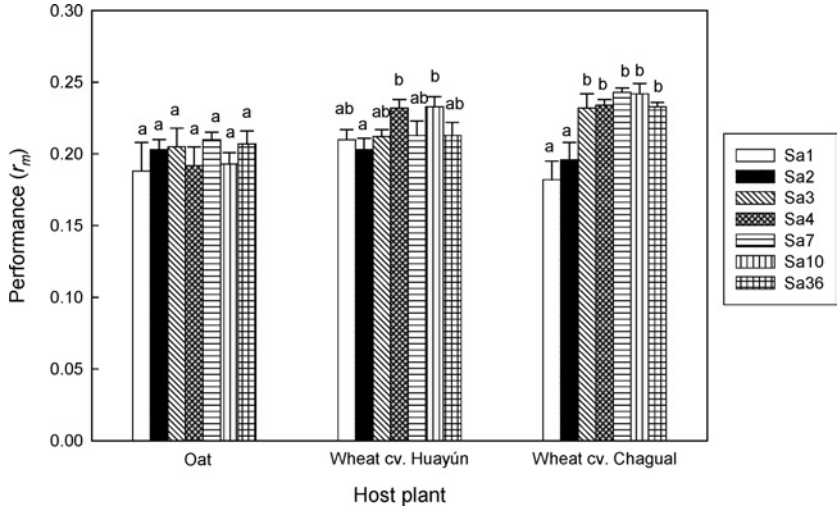

FIG. 2. Performances (r_m) of different genotypes of *Sitobion avenae* on oat (lacking hydroxamic acids—Hx; control), and wheat cultivars cv. Huayún (low-Hx) and cv. Chagual (high-Hx). Different letters indicate significant differences for intra-host comparisons (two-way ANOVA followed by LSD tests). The number of replicates performed for clones Sa1, Sa2, Sa3, Sa4, Sa7, Sa10, and Sa36 on the different hosts were as follows: oat (8, 13, 11, 9, 7, 11, 8), wheat cv. Huayún (12, 15, 12, 9, 9, 12, 10), and wheat cv. Chagual (12, 16, 10, 9, 10, 11, 10).

significant differences among several host plants (Table 1), exhibiting a higher prevalence on wheat and mouse barley than on oat, wild oat, or cocksfoot grass.

Chemical differences are known among the plant species studied. Wheat contains Hx, a family of secondary metabolites involved in deterrence and antibiosis against aphids (Niemeyer and Pérez, 1995), while *Hordeum* spp. may contain Hx (Barría et al., 1992) or gramine (Figure 1), an indolic alkaloid involved in resistance against aphids (Zúñiga and Corcuera, 1986; Gianoli and Niemeyer, 1998). These groups of metabolites are widely distributed among the Poaceae, but have not been detected in oat, wild oat, or cocksfoot grass (Niemeyer and Pérez, 1995; Niemeyer, unpublished data). Thus, plant chemical defenses and/or detoxification mechanisms in aphids may affect the clonal structure of *S. avenae* (Figueroa et al., 1999a; Loayza-Muro et al., 2000). Additionally, differences in the composition of RAPD-PCR phenotypes of *S. avenae* have been shown (Figueroa et al., 2002), with Hx affecting genetic variability in *S. avenae* populations.

These findings, in part, may explain the relative aphid distribution and abundance among hosts, as well as the nature of the most common genotypes of *S. avenae* found in Chile. Thus, genotype Sa1, the most abundant in Chile, showed

the highest frequency on hosts containing secondary chemistry that provides aphid resistance, i.e., wheat and mouse barley (Table 1). However, performance of genotype Sa1 was similar among host plants (Figure 2). The capacity of Sa1 to thrive on defended plants is a factor conferring on this genotype a particularly relevant colonizing ability. Additionally, the second most abundant genotype Sa2 was also observed with similar frequencies and performances on all host plants. These observations suggest that genotypes Sa1 and Sa2 could be the result of clonal selection promoting the evolution of general-purpose genotypes (Lynch, 1984), which would be characterized by a broad host range and a low variance for its performance on host plants with different defense chemicals levels (Figure 2).

Concerning the least common genotypes, the following model may account for their low frequency and their distribution and performance on different host plants. Introduction events of insect species have often been reported, and the low number of the individuals introduced initially leads to low genetic diversity of populations (genetic bottleneck) (Huey et al., 2000; Downie, 2002). Given the low genetic diversity and the strong clonal amplification in Chilean populations of *S. avenae*, it is likely that only a few clones were introduced into Chile some 30 years ago (Apablaza, 1974). The introduced clones were subjected, among other factors, to the selection pressure of Hx present in wheat, which occur at higher concentrations in cultivars sown in Chile than in Europe (Copaja et al., 1991; Nicol et al., 1992; Caillaud and Niemeyer, 1996), the likely region of origin of the introduced individuals (Figueroa et al., unpublished). In the absence or rarity of sexual reproduction (suggested by the predominance of a few multilocus genotypes along with departure from the Hardy–Weinberg equilibrium), emergence of new clones could occur not only as a consequence of new introductions, but also from spontaneous or induced point mutations (Dixon, 1998; Lushai et al., 2003; Wilson et al., 2003). Hydroxamic acids are mutagenic agents (Hashimoto et al., 1979; Hashimoto and Shudo, 1996), and induce the emergence of new clones within populations of *S. avenae* (Figueroa et al., 2002). New genotypes, initially at low frequencies, may have survived and even increased in relative abundance due to their enhanced performance on the best defended hosts. Indeed, some genotypes examined in this study other than the most frequent genotypes (likely, new genotypes) showed the highest performance on the host with the highest concentration of Hx (Figure 2). It is likely that these genotypes have acquired the capacity to detoxify allelochemicals such as Hx in a more efficient manner (Figueroa et al., 1999a; Loayza-Muro et al., 2000; Mukanganyama et al., 2003), or to sequester them more efficiently, as has been shown to occur in other aphid species (Wink and Romer, 1986). Based on the model presented herein, it is predicted that through time, these Hx-resistant clones will begin to prevail. Future assessments of the clonal composition of the Chilean populations of *S. avenae* will be necessary to test this prediction. Since other factors, such as climatic conditions, intraspecific competition, and reaction to natural enemies and to overwintering

refuges, can also constitute selective forces in the field, we propose Hx as an additional factor of population structuring. Further research will lead to a better understanding of the relative contributions of Hx and other factors to the genetic make-up of cereal aphid populations in the field.

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ACQUIRED AND R-GENE-MEDIATED RESISTANCE AGAINST THE POTATO APHID IN TOMATO

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Abstract—We examined the effects of three forms of host plant resistance in tomato, *Lycopersicon esculentum*, on the potato aphid, *Macrosiphum euphorbiae*. *Mi-1.2*, a resistance gene (R-gene) in tomato that deters aphid feeding, reduced the population growth of both potato aphid isolates tested, although it appeared to have a greater impact on isolate WU11 than on isolate WU12. The results suggest that there may be quantitative differences in virulence between these two aphid isolates. We also examined two distinct forms of acquired resistance in tomato, jasmonic acid (JA)-dependent and salicylic acid (SA)-dependent induced defenses. Exogenous foliar application of JA triggered expression of a JA-inducible proteinase inhibitor in tomato cultivars with and without *Mi-1.2*, although the effects of treatment on aphid performance differed between these cultivars. JA-treatment reduced aphid population growth on a susceptible tomato cultivar that lacks *Mi-1.2*, but did not significantly enhance or inhibit aphid control on a near-isogenic resistant tomato cultivar that carries this gene. Foliar application of an SA analog, benzothiadiazole (BTH), was used to induce SA-dependent defenses. BTH treatment reduced the population growth of both aphid isolates on a susceptible tomato cultivar, and also enhanced aphid control on a resistant cultivar. The results indicate that both SA- and JA-dependent acquired resistance in tomato have a direct negative effect on a phloem-feeding insect. Furthermore, this study demonstrates that acquired resistance and R-gene-mediated resistance can interact for enhanced suppression of insect herbivores.

Key Words—Insect resistance, induced resistance, systemic acquired resistance, *Mi*, *Meu1*, jasmonic acid, salicylic acid, benzothiadiazole, Homoptera, Aphididae.

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INTRODUCTION

Studies of host plant resistance typically have focused on two broad categories of plant defense acquired and innate resistance. Acquired resistance mechanisms, including systemic acquired resistance to pathogens and induced resistance to insects, are dependent upon systemic defenses that are induced by an initial pest infestation, and that render plants less susceptible to subsequent attack (Karban and Baldwin, 1997; Metraux et al., 2002). Furthermore, initial induction of these defenses by one pest can induce resistance to other attackers (Ryan et al., 1986; Stout et al., 1998a). In contrast, innate resistance targets specific pest species, and prevents or limits their initial establishment on the plant. Innate resistance, in many cases, depends upon single, dominant resistance genes, often termed R-genes (Hammond-Kossack and Jones, 1996). R-genes are thought to mediate gene-for-gene interactions with pests; in other words, a particular R-gene confers resistance against specific biotypes of pest that carry a corresponding avirulence (Avr) gene (Flor, 1955). The most extensively studied gene-for-gene interactions mediate resistance against plant pathogens, but R-genes are also thought to play a role in resistance against herbivores, including aphids, Hessian flies, gall midges, and nematodes (Puterka and Peters, 1989; Roberts, 1995; Zantoko, 1997; Milligan et al., 1998; Rossi et al., 1998; Stuart et al., 1998; Sardesai et al., 2001; Brotman et al., 2002).

Important signaling conflicts occur between different forms of acquired resistance. Induction of systemic acquired resistance to pathogens (SAR) can be inhibited by jasmonic acid, a signaling compound involved in induced resistance to chewing insects (Sano and Ohashi, 1995; Thaler et al., 1999). Likewise, salicylic acid, a key signal in SAR, can inhibit synthesis of jasmonic acid and suppress induction of induced resistance to insects (Pena-Cortes et al., 1993; Doares et al., 1995; Thaler et al., 1999). In contrast, little is known about the potential for signaling conflicts or synergisms between acquired and innate resistance, because these two broad categories of resistance have typically been studied separately. Certain studies, however, indicate that there is overlap between the physiological mechanisms that underlie acquired and innate resistance. For example, in addition to its role in SAR, salicylic acid is also required for the function of some but not all R-genes (Delaney et al., 1994; Glazebrook et al., 1996; Jirage et al., 1999; Brading et al., 2000; Kachroo et al., 2000; Rairdan and Delaney, 2002; Takahashi et al., 2002; Branch et al., 2004). Overexpression of certain R-genes also results in heightened SA levels and activation of SAR (Oldroyd and Staskawicz, 1998; Xiao et al., 2001). These findings suggest that SA-dependent systemic defenses and certain R-genes could potentially interact in a synergistic manner. For R-genes that are dependent upon SA for function, it is also possible that these genes might be incompatible with JA-dependent defenses.

The objectives of this study were to examine the effects of both acquired and innate resistance on an insect pest of tomato, and to determine if induction of acquired resistance would enhance or inhibit the effects of innate resistance. The two forms of acquired resistance examined in this study are SA- and JA-dependent systemic defenses. Relatively little is known about the impact of either of these defensive pathways on piercing–sucking insects, and so one goal was to evaluate their effects on the potato aphid, *Macrosiphum euphorbiae* Thomas. Our second goal was to examine potential interactions between these forms of acquired resistance and *Mi-1.2*, a source of innate aphid resistance in tomato, *Lycopersicon esculentum* Mill. *Mi-1.2* is a R-gene that confers resistance to certain isolates of the potato aphid, root-knot nematodes (*Meloidogyne* spp.), and the sweet potato whitefly (*Bemisia tabaci*) (Milligan et al., 1998; Rossi et al., 1998; Nombela et al., 2003). The physiological basis for *Mi*-mediated resistance is not yet well understood, but recent evidence indicates that SA is involved (Branch et al., 2004). *Mi*-mediated nematode resistance is inhibited by salicylate hydroxylase, a bacterial enzyme that degrades SA; conversely, nematode resistance can be restored to plants that express salicylate hydroxylase by treating the plants with a synthetic SA analog, benzothiadiazole (Branch et al., 2004). In addition, induction of *Mi*-mediated aphid resistance is correlated with the expression of marker genes associated with SA induction (de Ilarduya et al., 2003). Given that SA may play a role in *Mi*-mediated aphid resistance, one objective of this study was to determine if prior induction of SA-dependent defenses could enhance aphid resistance in a tomato cultivar that carries *Mi-1.2*. Furthermore, we wished to determine if induction of JA-dependent defenses would enhance or inhibit *Mi*-mediated aphid resistance.

METHODS AND MATERIALS

Plant Materials. Two near-isogenic tomato cultivars with and without *Mi-1.2* were used for our bioassays: Moneymaker (*Mi*−), and Motelle (*Mi*+). All plants were grown in 3.8-l pots of LC1 Sunshine potting mix (Sungro Horticulture, Bellevue, WA) under stable greenhouse conditions ($\sim$ 24°C–27°C; 16:8 L:D photoperiod). Plants were watered daily with a dilute nutrient solution containing 1000 mg/l $CaNO_3$ (Hydro Agri North America, Tampa, FL), 500 mg/l $MgSO_4$ (Giles Chemical Corp, Waynesville, NC), and 500-mg/l Hydroponic 4-18-38 Growmore fertilizer (Growmore, Gardena, CA).

Insect Cultures. Two potato aphid isolates, which we designated WU11 and WU12, were utilized. Each was a clonal population established from a single female. Isolate WU11 originated from the laboratory colony of Dr. Yvan Rahbe, which was originally maintained on eggplant (*Solanum melongena*) (Goggin et al.,

2001). Aphid isolate WU12 was obtained from the laboratory colony of Dr. Stuart Seah, and was originally maintained on potato (*Solanum tuberosum*) (Goggin et al., 2004). Both isolates were maintained on susceptible tomato seedlings (cv. UC82) for more than 2 yr prior to these experiments, to insure that both were well adapted to tomato. Aphids were maintained in Conviron growth chambers (Controlled Environments, Inc., Winnipeg, Canada) under optimal conditions for aphid development (20°C, 16:8 L:D photoperiod).

Benzothiadiazole Application. SA-dependent defenses were induced in tomato by applying a foliar treatment of benzothiadiazole (BTH), a synthetic analog of SA. Exogenous treatments of SA and BTH have both been shown to induce acquired resistance to pathogens and induction of associated pathogenesis-related proteins (Friedrich et al., 1996; Gorlach et al., 1996; Lawton et al., 1996). We chose to use BTH rather than SA as a defense elicitor because it lacks the phytotoxic effects associated with SA, and has stronger systemic effects than exogenous SA (Friedrich et al., 1996).

BTH (Syngenta Crop Protection, Greensboro, NC) was dissolved in acetone at a rate of 28 g/l and dispersed in water to achieve a 1.2 mM BTH solution (Friedrich et al., 1996). For the control treatments, an equal quantity of acetone (without BTH) was dispersed in water. Approximately 6 wk after germination, at which stage *Mi*-mediated resistance is active in the foliage, tomato plants were sprayed with BTH solution or control solution applied at a rate of 1 ml per leaf using an atomizer (~12 ml/plant). The eighth leaf from the cotyledon of each plant was protected from treatment with a plastic bag, which was removed when all other leaves were dry. Aphid bioassays (described below) were performed using this untreated leaf, so that we could measure the effects of acquired resistance on aphids independent of any effects that BTH residue might have on the insects.

Jasmonic Acid Application. JA-dependent defenses were induced in tomato by applying a foliar treatment of exogenous JA, as previously described by Thaler et al. (1999). Jasmonic acid (Sigma Chemicals, St Louis, MO) was dissolved in acetone at a rate of 1 g/ml and dispersed in water to achieve a 1.5 mM JA solution (Thaler, 1999). An equal quantity of acetone was dispersed in water (without JA) for control treatment. JA and control treatments were applied 6 wk after germination as described for BTH. One leaf per plant was protected from treatment for use in aphid bioassays.

Aphid Bioassays. In trial 1, the effects of BTH on population growth of aphid isolates WU11 and WU12 were measured by using two independent bioassays. In each assay, aphid performance was measured on Moneymaker (*Mi*−) or Motelle (*Mi*+) sprayed with either BTH or control solution. Forty-eight hr after treatment, the terminal leaflet of the eighth leaf from the cotyledon was inoculated with 15 aphids, which were confined to the leaflet with a sleeve cage (12 plants/treatment for the WU11 assay; 8 plants/treatment for the WU12 assay). Plants were inoculated with a combination of fourth-instar juveniles and young

adult aphids, and the ratio of adults to juveniles was standardized from plant to plant. Six days after inoculation, aphid performance was evaluated by counting the total number of aphids/cage. Bioassays were performed in a Conviron growth chamber (20°C; 16:8 L:D photoperiod).

In trial 2, the effects of BTH treatment on WU11 performance on Motelle (*Mi*+) were examined further. Plants were treated with BTH or control solution as described above, and inoculated with 15 fourth-instars confined to a single sleeve cage per plant (9 plants/treatment). Six days after inoculation, aphid performance was evaluated by counting the number of aphids that survived to adulthood and the number of offspring they produced. Adults and juveniles were recorded separately in order to detect any subtle effects on aphid survivorship or fecundity that might not be apparent from total aphid numbers.

The effects of JA on WU11 and WU12 were tested in the same manner as described above for trial 1 of the BTH study (13 plants/treatment for the WU11 assay; 9 plants/treatment for the WU12 assay). Plants sprayed with JA vs. control treatments were maintained in separate growth chambers to insure that volatiles from plants treated with JA would not affect control plants.

For each assay, foliar application and genotype were compared as independent fixed factors by full factorial two-way ANOVA using JMP version 5.01 (SAS, Cary, NC). Treatment combinations were analyzed by Tukey's HSD statistics and paired *t*-tests using JMP version 5.01.

Analysis of Gene Expression. To determine if JA-dependent defenses are induced equally in different tomato cultivars, expression of *Proteinase Inhibitor II* (*Pin2*) was measured on Motelle and Moneymaker tomato using reverse transcriptase-polymerase chain reaction (RT-PCR). Motelle and Moneymaker plants were treated with a foliar application of synthetic JA or control solution as described above. Forty-eight hr after treatment, ~1 g of leaf tissue was collected from one untreated leaf per plant (2 reps/treatment) and flash frozen with liquid nitrogen. RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and DNase-treated with DNA-*free* reagents (Ambion, Austin, TX) according to manufacturers' protocols. Aliquots of RNA were quantified using a spectrophotometer and run on an agarose/formaldehyde gel to check quality. cDNA was synthesized from 0.4 μg of total RNA using a RetroScript Reverse Transcriptase kit and oligo-DT primers (Ambion, Austin, TX). To amplify *Pin2* cDNA, the following primers were used: *Pin2* forward, 5′-CCG TTC ACA AGG AAA ATC GT-3′, and reverse, 5′-ATT TTG GGC AAT CCA GAA GA-3′. The size of the amplified fragment was 186 bp. In separate PCR reactions, expression of the housekeeping gene ubiquitin III (*Ubi3*) was measured using the following primers: *Ubi3* forward 5′-GTG TGG GCT CAC CTA CGT TT-3′, and reverse, 5′-ACA ATC CCA AGG GTT GTC AC-3′. The size of the amplified fragment was 162 bp. PCR with *Ubi3* primers was also performed on total RNA from each sample to confirm that the samples did not contain genomic DNA (RT − controls). PCR was performed by using an MJ

Research PTC-200 thermal cycler (MJ Research, San Francisco, CA). Conditions for all PCR reactions were 4 min at 95°C, 24 cycles (94°C, 30 sec; 57°C, 30 sec; 72°C 30 sec) followed by 5 min at 72°C. PCR products were visualized by gel electrophoresis with a 2% agarose gel.

RESULTS

Effects of BTH and Mi-1.2 on Aphids. In trial 1, WU11 aphid numbers after 6 days of feeding differed among treatment groups (Figure 1) ($F = 26.14$; $df = 3$; $P < 0.001$). There was no significant interaction between plant genotype and foliar treatment ($F = 1.32$; $df = 1$; $P = 0.265$). Aphid numbers were lower on Motelle ($Mi+$) plants compared to Moneymaker ($Mi-$) ($F = 69.7$; $df = 1$; $P < 0.001$), which confirms previous findings that WU11 is impacted by *Mi-1.2* (Goggin et al., 2001). Aphid numbers were also reduced by BTH application

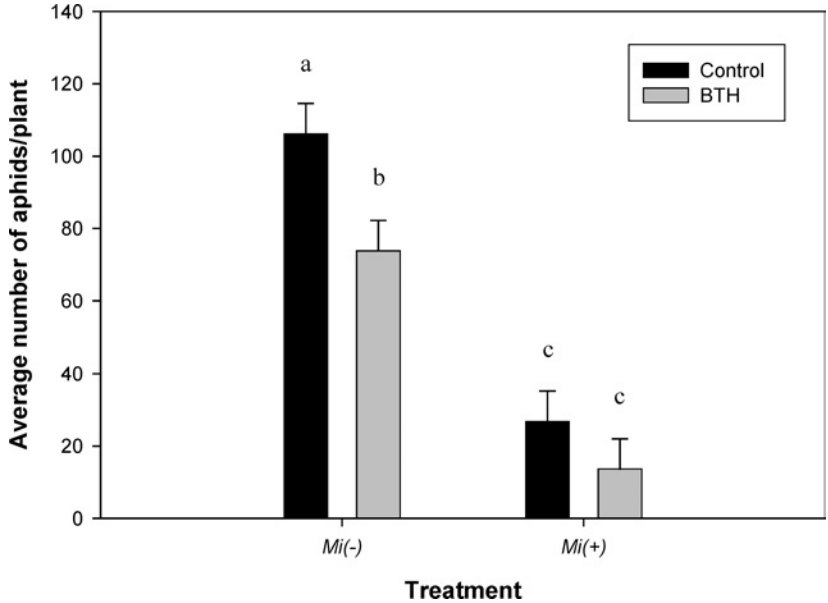

FIG. 1. *Effects of BTH Treatment and Mi-1.2 on Aphid Isolate WU11.* Near-isogenic tomato plants with and without *Mi-1.2* were sprayed with a 1.2 mM solution of BTH, or with a control solution. Forty-eight-hr after treatment, one leaflet per plant was inoculated with 15 WU11 aphids each. Total aphid numbers were measured 6 days after inoculation. Error bars represent the standard deviations. Values with differing letters are significantly different at the $\alpha = 0.05$ confidence interval according to Tukey HSD statistics.

TABLE 1. EFFECTS OF BTH TREATMENT ON APHID ISOLATE WU11 PERFORMANCE ON A RESISTANT (*Mi*+) TOMATO CULTIVAR[a]

	Adults/Plant ± SE	Juveniles/Plant ± SE	Total/Plant ± SE
Control	5 ± 3 A	18 ± 6 A	23 ± 7 A
BTH	4 ± 2 A	10 ± 7 B	14 ± 8 B

[a] Within each column, values labeled with different letters are significantly different at $\alpha = 0.05$ according to a paired t-test.

($F = 7.42$; $df = 1$; $P = 0.013$). BTH treatment dramatically reduced aphid numbers on the susceptible cultivar Moneymaker ($t = -2.74$; $df = 1$; $P = 0.013$). On the resistant cultivar, aphid numbers were lower on BTH-treated plants than on control plants, but this difference was not significant according to a paired t-test ($t = -1.114$; $df = 1$; $P = 0.278$). In trial 2 (Table 1), BTH treatment reduced total WU11 aphid numbers on the resistant cultivar Motelle ($F = 5.852$; $df = 1$; $P = 0.030$). This effect appeared to be due to a reduction in aphid reproduction on plants treated with BTH. While the number of surviving adults did not differ between treatments ($F = 0.221$; $df = 1$; $P = 0.64$), the number of juveniles on BTH-treated plants was lower compared to controls ($F = 7.215$; $df = 1$; $P = 0.018$).

WU12 aphid numbers after six days of feeding differed among treatments (Figure 2) ($F = 1.44$; $df = 3$; $P < 0.001$). There was no significant interaction between *Mi*-mediated resistance and BTH application ($F = 2.08$; $df = 1$; $P = 0.161$). Aphid numbers were lower on Motelle vs. Moneymaker plants ($F = 2.89$; $df = 1$; $P = 0.008$), which confirms previous findings that *Mi-1.2* is active against aphid isolate WU12 (Goggin et al., 2004). Aphid numbers were also lower on plants treated with BTH compared to control plants ($F = 4.93$; $df = 1$; $P < 0.001$). BTH treatment reduced aphid numbers on both Moneymaker and Motelle ($t = -4.588$; $df = 1$; $P < 0.001$ and $t = -2.426$; $df = 1$; $P = 0.022$, respectively).

Although both aphid isolates were impacted by *Mi-1.2*, this resistance gene appeared to have a stronger effect on WU11 than on WU12. WU11 numbers on control Motelle plants (untreated with JA) were 74.7% lower than WU11 numbers on control Moneymaker plants (Figure 1); in contrast, WU12 numbers on Motelle control plants were only 33% lower than WU12 numbers on Moneymaker control plants (Figure 2).

Effects of JA and Mi-1.2 on Aphids. WU11 aphid numbers differed among treatments after 6 days of feeding (Figure 3) ($F = 268.92$; $df = 3$; $P < 0.001$). There was a significant interaction between plant genotype and foliar application ($F = 7.57$; $df = 1$; $P = 0.008$), because the effects of JA treatment differed between genotypes. Therefore, the effects of JA and plant genotype were analyzed

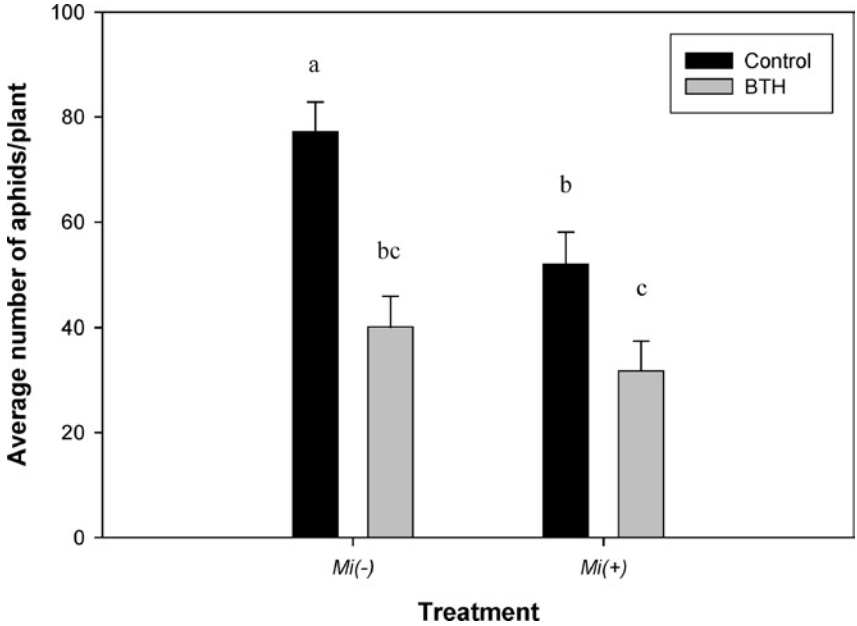

FIG. 2. *Effects of BTH Treatment and Mi-1.2 on Aphid Isolate WU12*. Near isogenic tomato plants with and without *Mi-1.2* were sprayed with a 1.2 mM solution of BTH, or with a control solution. Forty-eight hr after treatment, one leaflet per plant was inoculated with 15 WU12 aphids each. Total aphid numbers were measured 6 days after inoculation. Error bars represent the standard deviations. Values with differing letters are significantly different at the $\alpha = 0.05$ confidence interval according to Tukey HSD statistics.

separately. Comparisons between Moneymaker and Motelle plants sprayed with the control treatment revealed that *Mi-1.2* had a strong negative effect on aphid numbers ($t = 21.88$; $df = 1$; $P < 0.001$). JA treatment reduced aphid numbers on the susceptible cultivar Moneymaker ($t = 3.41$; $df = 1$; $P = 0.001$), but did not influence aphid numbers on the resistant cultivar Motelle ($t = 0.419$; $df = 1$; $P = 0.632$).

WU12 aphid numbers after 6 days of feeding differed among treatments (Figure 4) ($F = 5.24$; $df = 3$; $P > F = 0.049$). There was no interaction between genotype and JA application ($F = 2.093$; $df = 1$; $P = 0.158$). The overall effect of the plant genotype was not significant at $\alpha = 0.05$ confidence interval ($F = 3.24$; $df = 1$; $P = 0.082$), although aphid numbers were lower on Motelle vs. Moneymaker control plants ($t = 3.33$; $df = 1$; $P = 0.002$). Aphid numbers were reduced on plants sprayed with JA compared to control plants ($F = 11.14$; $df = 1$; $P = 0.002$), although JA did not enhance aphid control on the resistant cultivar Motelle ($t = 1.36$; $df = 1$; $P = 0.185$).

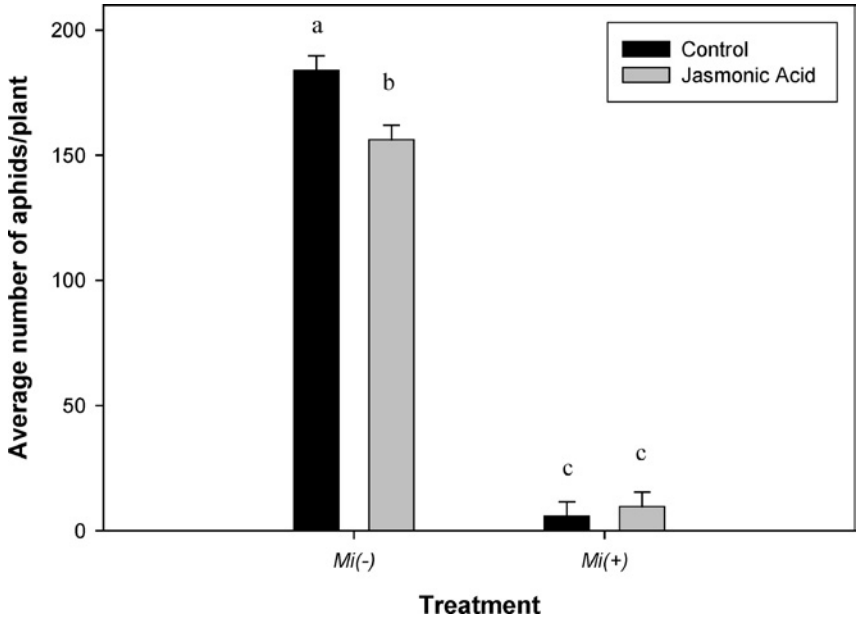

FIG. 3. *Effects of JA Treatment and Mi-1.2 on Aphid Isolate WU11*. Near isogenic tomato plants with and without *Mi-1.2* were sprayed with a 1.5 mM solution of JA, or with a control solution. Forty-eight hr after treatment, one leaflet per plant was inoculated with 15 WU11 aphids each. Total aphid numbers were measured 6 days after inoculation. Error bars represent the standard deviations. Values with differing letters are significantly different at the $\alpha = 0.05$ confidence interval according to Tukey HSD statistics.

Similar to our bioassays with BTH, our assays with JA indicated that aphid isolates WU11 and WU12 appear to differ in their response to *Mi*-mediated resistance. When we compare aphid numbers on untreated Moneymaker vs. untreated Motelle plants within each assay, we find that WU11 numbers were reduced by >99% on Motelle (Figure 3), whereas WU12 numbers were reduced by only 24.2%.

Induction of Pin2 Expression. Because we were unable to detect a significant biological effect of JA application on Motelle plants, we performed RT-PCR to confirm that foliar application of JA could induce JA-dependent defenses in this genotype. Expression of the *Pin2* gene has been shown to be a reliable marker of induction of JA-dependent defenses (Graham et al., 1985; Stout et al., 1998b), and so we compared *Pin2* expression in Moneymaker and Motelle plants treated with JA or control solution. *Pin2* transcripts were detected in comparable abundance in Moneymaker and Motelle plants sprayed with JA, and were absent in plants treated with control solution (Figure 5). These results indicate that foliar

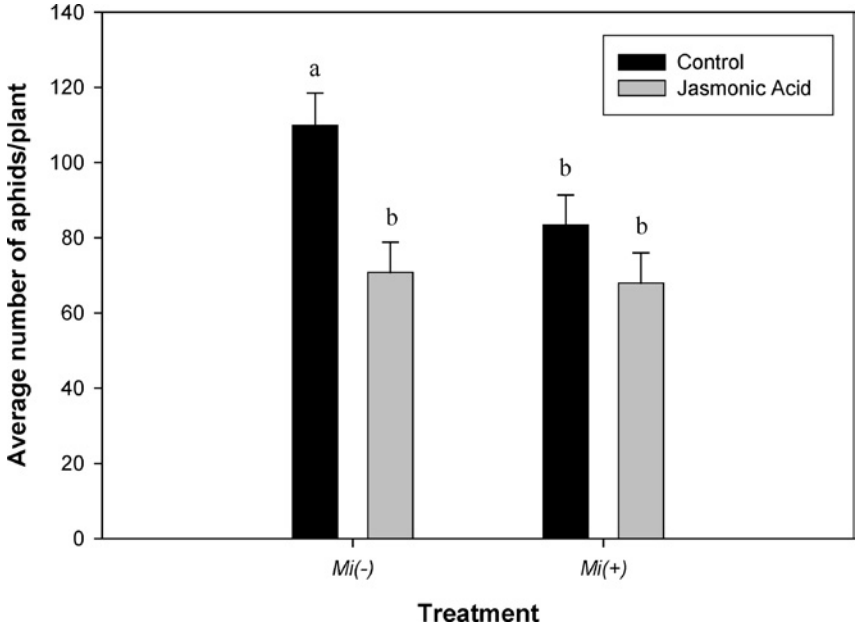

FIG. 4. *Effects of JA Treatment and Mi-1.2 on Aphid Isolate WU12*. Near-isogenic tomato plants with and without *Mi-1.2* were sprayed with a 1.5 mM solution of JA, or with a control solution. Forty-eight hr after treatment, one leaflet per plant was inoculated with 15 WU12 aphids each. Total aphid numbers were measured 6 days after inoculation. Error bars represent the standard deviations. Values with differing letters are significantly different at the $\alpha = 0.05$ confidence interval according to Tukey HSD statistics.

application of JA induced proteinase inhibitor expression in both genotypes tested. The housekeeping gene ubiquitin III was uniformly expressed in all samples, confirming that these samples contained comparable amounts of mRNA (Figure 5). No amplification products were detected when our RNA samples were used as a template for PCR (Figure 5). This indicates that our RNA samples were free from contaminating genomic DNA.

DISCUSSION

This study demonstrates that both SA- and JA-dependent defenses reduce potato aphid populations on a susceptible (*Mi*−) tomato cultivar. The results also indicate that artificial induction of SA-dependent defenses can enhance aphid control on a resistant (*Mi*+) cultivar. In contrast, induction of JA-dependent defenses did not appear to enhance or inhibit *Mi*-mediated aphid resistance.

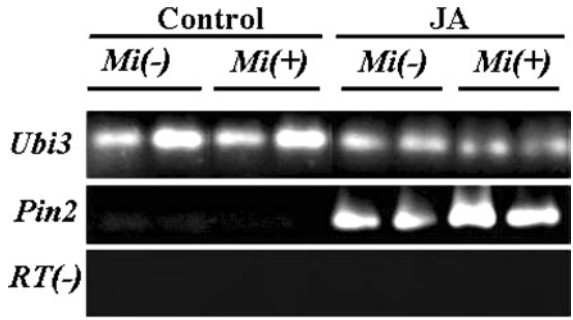

FIG. 5. *Induction of Pin2 Expression*. RT-PCR was used to compare expression of proteinase inhibitor II (*Pin2*), which is associated with induction of JA-dependent defenses, in Moneymaker (*Mi*−) and Motelle (*Mi*+) plants treated with control solution or 1.5 mM JA. Expression of the housekeeping gene ubiquitin III (*Ubi3*) was analyzed to verify that all samples contained comparable amounts of mRNA. PCR with ubiquitin primers was also performed on RNA aliquots (RT − samples) to confirm that the RNA samples were not contaminated with genomic DNA.

These findings add to a growing body of evidence that SA may play a role in induced resistance to piercing-sucking insects. Although induction of SA and related compounds is typically associated with plant defenses against pathogens, recent studies indicate that aphid infestation triggers SA accumulation in barley and wheat, and that psyllids elicit methyl salicylate production in pear (Havlickova et al., 1998; Mohase and van der Westhuizen, 2002; Chaman et al., 2003; Scutareanu et al., 2003). Aphid and whitefly feeding on a variety of plant species also has been shown to induce pathogenesis-related (PR) proteins associated with SA induction, such as glucanases, chitinases, and peroxidases (Krishnaveni et al., 1999; Forslund et al., 2000; Mayer et al., 2002; Moran et al., 2002; de Ilarduya and Delany, 2003; Zhu-Salzman et al., 2004). By comparing cultivars that vary in their susceptibility to aphids, some of these studies have demonstrated a positive correlation between levels of aphid resistance and levels of SA or PR protein induction (Forslund et al., 2000; Chaman et al., 2003; de Ilarduya et al., 2003). To our knowledge, however, only one prior study has directly demonstrated that SA-dependent defenses impact aphid fitness. In this case, a modest reduction in green peach aphid numbers was observed on *Arabidopsis* foliage treated with benzothiadiazole (BTH), a functional analog of SA (Moran and Thompson, 2001). Our study demonstrates that BTH also induces systemic defenses that dramatically reduce potato aphid population growth on a susceptible tomato cultivar (Figures 1 and 2; Table 1). Furthermore, BTH treatment can enhance R-gene mediated aphid resistance in tomato (Table 1; Figure 2). This enhanced aphid control may be due to an additive effect of BTH-induced defenses and *Mi*-mediated resistance. Alternatively, the defense response mediated

by *Mi-1.2* may be faster or stronger in plants that have previously been conditioned by BTH treatment.

Interestingly, the impact of *Mi*-mediated resistance appeared to differ between aphid isolates WU11 and WU12. This finding is intriguing because it suggests that virulence could potentially be a quantitative rather than a qualitative trait. WU11 and WU12 both establish significantly higher numbers on susceptible vs. resistant plants; therefore, they are both classified as avirulent (Goggin et al., 2001, 2004). Nonetheless, *Mi-1.2* appears to have a greater impact on the population growth of WU11 than on WU12. This suggests that the outcome of the plant-insect interaction is influenced by factors other than the simple presence or absence of a single avirulence factor. The extreme sensitivity of WU11 to *Mi*-mediated resistance may also explain why BTH treatment did not always enhance control of WU11 on resistant plants (Figure 1). *Mi-1.2* suppresses WU11 population growth so effectively that the impact of further control measures such as BTH is often not statistically significant.

This study also investigated the effects of JA-dependent defenses on the potato aphid. Most previous studies of JA have focused on its role in induced resistance against caterpillars and other chewing insects. Artificial induction of JA, however, is also known to affect aphids (Thaler et al., 1999; Omer et al., 2001; Ellis et al., 2002; Zhu-Salzman et al., 2004). In tomato, for example, foliar application of JA aphid reduces infestations under field conditions (Thaler et al., 1999). This reduction may have been due to an effect of JA or JA-dependent defenses on aphid migration, aphid population growth, or rates of predation and parasitism. In this study, we demonstrate that systemic defenses induced by JA in susceptible tomato plants have a negative impact on aphid population growth in the absence of natural enemies or aphid migration (Figures 3 and 4). In contrast, JA treatment did not dramatically enhance aphid control on a resistant tomato cultivar that carries *Mi-1.2* (Figures 3 and 4). This finding is also supported by the results of a life table study, in which we found that artificial induction of JA-dependent defenses reduced the longevity and lifetime fecundity of potato aphids on susceptible, but not on resistant, tomato plants (Cooper and Goggin, unpublished data).

It is not yet clear why JA treatment does not enhance aphid control on resistant varieties. Exogenous JA induced strong expression of the *Pin2* gene in both the resistant and susceptible cultivar, which suggests that treatment induces JA-dependent defenses in both genotypes (Figure 5). Potentially, the effects of *Mi-1.2* may prevent these JA-dependent defenses from having a dramatic impact on aphids. *Mi*-mediated resistance dramatically reduces aphid feeding (Kaloshian et al., 1997), whereas JA-dependent defenses appear to have primarily antibiotic effects on aphids (Cooper and Goggin, unpublished data). By reducing aphid feeding, *Mi-1.2* could thereby reduce aphids' exposure to toxic or antinutritive defenses induced by JA. In other words, a difference in the mode of action of these two forms of resistance could prevent them from having additive effects. While JA

treatment did not enhance *Mi*-mediated resistance, it is important to note that it also did not render plants more susceptible to aphids. This is significant because signaling conflicts can in some cases occur between SA- and JA-dependent defensive pathways, and SA is thought to play an important role in *Mi*-mediated resistance. Potentially, plants that carry *Mi-1.2* could be treated with JA to control other pests such as caterpillars, without compromising *Mi*-mediated aphid resistance.

Plant activators that elicit SA- or JA-dependent defenses are used in commercial tomato production to control pathogens and other pests, and these activators represent an environmentally safe alternative to pesticides. The results from this study suggest that artificial induction of these defensive pathways could also protect susceptible tomato cultivars against potato aphid infestation. This finding is of practical significance because the potato aphid is a major pest of tomato (Walgenbrach, 1997), and many widely used commercial tomato varieties are susceptible to this pest. In addition, this study demonstrates that induction of SA-dependent defenses could enhance aphid control on resistant tomato varieties that carry *Mi-1.2*. Aphid isolates that can overcome *Mi*-mediated resistance have been identified in the US (Goggin et al., 2001), and BTH or other elicitors of SAR could potentially help control these virulent biotypes. We hope that this work contributes to the utilization of host plant defenses for pest management, as well as to our understanding of the interactions between acquired and innate resistance.

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MAIZE GENES INDUCED BY HERBIVORY AND VOLICITIN

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Abstract—In crop plants, both mechanical damage and insect attack trigger rapid changes in gene transcription. We investigated whether insect herbivory differs from a general wound response, and if so, is the induction specific to the pest/host plant interaction? Herbivory by beet armyworm (BAW; *Spodoptera exigua*) caterpillars on maize results in a unique pattern of volatile compounds not triggered by wounding alone that attracts the generalist parasitoid *Cotesia marginiventris*. Caterpillar-induced volatile emission can be mimicked when a component of the BAW oral secretions (*N*-(17-hydroxylinolenoyl)-L-glutamine) termed volicitin, is applied to wounded leaves. We identified genes that are affected by BAW feeding by comparing volicitin treatment with wounding alone. We compared cDNAs from these two populations by isolating genes from a subtractive library and using reverse northerns. Virtual northern blots confirmed these results and further showed that BAW infestation affected the expression of these genes. In some cases, BAW feeding inhibited the expression of volicitin-induced genes, suggesting the role of additional bioactive components in caterpillar regurgitate. Transcripts involved in volatile production are increased by volicitin and BAW infestation treatments, and are also detectable at low levels in mechanically wounded leaves. Finally, we identified three new sesquiterpene cyclase genes that are induced by volicitin.

Key Words—Insect herbivory, maize, plant–insect interaction, volicitin.

INTRODUCTION

Herbivory induces an array of genes, including those responsible for direct and indirect defense responses. Direct defenses are commonly metabolites that interfere with insect feeding and nutritions (Kessler and Baldwin, 2002), such as proteinase inhibitors that inactivate digestive enzymes, and polyphenol oxidase, which crosslinks plant proteins making them less nutritions. Indirect defenses

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occur when products from the infested plant attract natural enemies of the attacking insect (Dicke, 1999). For example, infestation of maize with beet armyworm (BAW; *Spodoptera exigua*) causes the production of volatile compounds that attract the generalist parasitoid *Cotesia marginiventris* to the BAW larval host (Turlings et al., 1990). After oviposition, the parasitoids develop inside the lepidopteran larvae, killing them, reducing the level of herbivory, and preventing further pest reproduction (Hoballah and Turlings, 1999). A wide range of plant species such as apple, lima bean, and cotton produce similar volatile compounds in response to insect herbivory (Pare and Tumlinson, 1999).

Induced volatile emission following mechanical damage and BAW herbivory differ greatly, and enables efficient searching by parasitoids. However, the profile of volatiles produced by the infested leaf can be mimicked if a mechanically damaged leaf is treated with volicitin, a fatty acid-amino acid conjugate (FAC) in BAW regurgitant (Alborn et al., 1997). In addition to volicitin, several classes of elicitors from insect oral secretions have been described. A β-glucosidase from *Pieris brassicae* (cabbage-white butterfly) (Mattiacci et al., 1995), Glc oxidase from *Helicoverpa zea* (corn earworm) (Musser et al., 2002), and additional FACs from lepidopteran species (Pohnert et al., 1999; Halitschke et al., 2001) all alter plant responses when applied to a wounded leaf.

Evidence exists predicting that the increase of certain volatiles is a result of *de novo* protein synthesis (Pare and Tumlinson, 1997), suggesting an increase in transcript levels of genes associated with this process. In fact, the gene *Igl*, which encodes an enzyme that produces the volatile indole, is induced quickly upon addition of volicitin to wounded tissue (Frey et al., 2000). The gene products of *TPS1* and *Stc1* produce volatiles sesquiterpene (Shen et al., 2000; Schnee et al., 2002), and the steady state level of the transcript is higher following insect herbivory and elicitor application.

To clarify further the transcriptional basis of insect-specific defense responses, we set out to isolate genes that are differentially affected by insect elicitors. This was accomplished by the production of a subtractive cDNA library in which RNA from mechanically damaged tissue was subtracted from RNA that had been extracted from tissue that had been wounded and treated with volicitin. By using excessive wound-induced RNA, genes that are particularly sensitive to herbivore elicitors rather than mechanical damage, a non-specific component of insect herbivory, are preferentially cloned. Generally we found that genes involved in volatile production were induced and isolated three putative sesquiterpene cyclase genes.

METHODS AND MATERIALS

Plant Growth Condition. Seeds of *Zea mays* cv Delprim were acquired from Delley Seeds (Delley, Switzerland). Plants were germinated in vermiculite,

grown for 6 d, and then transferred to hydroponic containers (see Schmelz et al., 2001). The hydroponic solution was complete except for limiting levels of NO_3^- (0.2 mM) as described in Schmelz et al. (2003a). These low N conditions increase plant sensitivity and induced volatile responses to volicitin.

Physical and Chemical Leaf Treatments. For mechanical damage treatments, each of the oldest three leaves of individual plants received two superficial damage sites by using a razor to scratch the abaxial surface of the leaves perpendicular to but not including the midrib vasculature. The mechanical damage sites (normally 2 × 10 mm) were approximately equidistant between the base and tip of the leaf but laterally staggered by 2 cm with one on each side of the midrib. This treatment disrupted the waxy cuticle and epidermal cells and allowed applied buffer solutions to cling to the leaf surface. A total of 10 μl of 50 mM sodium phosphate (pH = 8.0) buffer were distributed evenly between all mechanical damage sites on each plant immediately after wounding. The quantity of volicitin dissolved in buffer and applied to each plant was 5 nmol. Leaf treatments were performed immediately before the end of the photophase (6 p.m.). In corn, BAW herbivory induces ethylene emission, but mechanical damage volicitin treatments do not (Schmelz et al., 2003b). To better mimic insect attack, both wounded and wound + volicitin treated plants were additionally exposed to 50 nl L^{-1} ethylene during the overnight incubation period as described in Schmelz et al. (2003a). Intact plants were placed in sealed 7 L cylindrical Plexiglas™ chambers (12 × 62 cm) fitted with rubber septa for the introduction of ethylene. Ethylene (Scotty® II Gases, Alltech, Deerfield, IL) addition was always performed immediately after elicitor treatments.

Analyses of Volatiles. Collection and gas chromatography analysis of volatiles were performed as described in Schmelz et al. (2001). Plant volatiles were collected for 1 hr at 12 hr after treatment. This period corresponds with maximal volatile emission (Schmelz et al., 2001, 2003c). The three major insect-induced sesquiterpenes (cs) in maize (var Delprim) are β-caryophyllene, (*E*)-α-bergamotene, and (*E*)-β-farnesene.

RNA Isolation and Subtractive Library Production. RNA was isolated from tissue frozen in liquid nitrogen and ground to a fine powder using the procedure described in Chang et al. (1993). Maize tissue was extracted with vigorous shaking in a buffer preheated to 65°C containing 2% CTAB, 2% polyvinylpyrrolidone, 100 mM Tris pH 8.0, 25 mM EDTA, 2 M NaCl, 0.5 g/l spermidine, and 2% β-mercaptoethanol. This mixture was chloroform extracted. The aqueous layer was precipitated at 4°C overnight in an ice water bath with 1/4 volume of 10 M LiCl. The pellet was collected by centrifugation at 11,950 × g for 20 min and resuspended in 1.0 M NaCl, 0.5% SDS, 10 M Tris pH 8.0, and 1 M EDTA. RNA was extracted once with chloroform and precipitated in two volumes of 95% ethanol.

In order to preferentially clone volicitin-induced genes, a subtractive cDNA library was produced using the PCR-Select cDNA subtraction kit[2] from Clontech (Palo Alto, CA) following the procedure outlined by Diatchenko et al. (1996). Double stranded cDNA was synthesized using a SMART™ PCR cDNA Synthesis kit from Clontech. An excess of cDNA from wounded material was hybridized to cDNA from wounded + volicitin-treated material, which enriches volicitin-induced cDNAs. The resulting cDNAs were cloned into pGem-T using Vector system I from Promega (Madison, WI).

Differential Screening of the Subtracted Library. The cDNA clones were amplified by PCR with kit-specific primers (nested primer 1 and nested primer 2R), and the DNA from each clone was mixed with an equal volume of 0.6 N NaOH and spotted onto nylon membrane in a 96-well format. The blots were neutralized in 0.5 M Tris–HCl (pH 7.5) and washed in water. The DNA was baked onto the blots for 2 hr in a 70°C oven. The dot blots were hybridized with either ^{32}P random labeled volicitin-induced enriched cDNA or wound-induced enriched cDNA probe following a protocol for screening a cDNA subtractive library described in the PCR-select differential screening kit manual from Clontech. The volicitin-induced enriched probe was prepared by hybridizing an excess of wound-induced cDNA from volicitin-induced cDNA, thus enhancing volicitin-induced genes. The wound-induced enriched probe was prepared by hybridizing an excess of volicitin-induced cDNA to wound-induced cDNA, enhancing genes that were induced differentially in the wounded tissue. The probes were labeled with ^{32}P using the Prime-It II random primer labeling kit from Stratagene (Cedar Creek, TX).

Virtual Northern Blots and Generation of Longer cDNAs of Sesquiterpene Synthases. Virtual northerns were performed as described in Endege et al. (1999) using a protocol included in the cDNA synthesis kit from Clontech. This procedure is more sensitive than a standard Northern in that it uses cDNA rather than RNA. cDNA from different RNA samples was made using a poly-T primer along with annealing of a 5′ universal primer for PCR amplification of the cDNA. The amplified cDNA was loaded and run on an agarose gel, and capillary transfer of the cDNA was performed (Sambrook et al., 1989). Nested primers 1 and R2 were used to amplify selected clones to be labeled with ^{32}P using the Prime-It II random primer labeling kit from Stratagene. Virtual northern blots were hybridized and washed using the ExpressHyb hybridization solution from Clontech.

Since the initial clones were not full length, 3′ and 5′ ends of sesquiterpene synthase clones were generated using 5′ and 3′ RACE kits from Invitrogen (Carlsbad, CA) following a protocol outlined by Frohman (1990). Using a

[2]Disclaimer: Mentioning of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

gene-specific primer that binds approximately 300 bp from the known 5′ end of the RNA, cDNA was produced using reverse transcriptase. The RNA was then degraded with RNase, and the single stranded cDNA was tailed with deoxycytidine using terminal deoxynuceotidyl transferase. The tailed cDNA was made double stranded by PCR amplification using a kit-specific primer that bound to the polyC tail and a gene-specific primer that bound 3′ of the first gene specific primer site. Finally, the cDNA was amplified and made more specific by PCR amplification using a third gene-specific primer and a kit-specific primer that binds to the 5′ end. The 3′ ends of clones were produced by making single stranded RNA using an oligo(dT) kit specific primer. The RNA was degraded with RNase H, and the cDNA was made double stranded by PCR amplification using a gene-specific primer and a primer that binds to the oligo(dT) kit-specific primer. The PCR reaction was run on a 1% agarose gel in TBE, and a pipette tip was used to isolate a piece of the ethidium bromide-stained band. The gel piece was used in a final PCR reaction using a second gene-specific primer and a kit-specific primer that binds to the 3′ end. The cDNAs were cloned into pGem-T using Vector system I from Promega (Madison, WI).

Sequence Analysis. Clones that were either induced or inhibited by the addition of volicitin to the tissue were selected and sequenced using Bigdye terminator sequencing by Applied Biosystems (Foster City, CA) using a 3100 ABi Prism sequencer. DNA sequences were analyzed using DNASTAR. Sequences were compared with the database at the National Center for Biotechnology Information using tBLASTx and BLASTx (Altschul et al., 1997). A phylogenetic tree for the cloned sesquiterpene synthases was generated using neighbor joining and distance programs of PAUP (Swofford, 1998. Version 4. Sinauer Associates, Sunderland, MA).

RESULTS AND DISCUSSION

Volatile Production. Addition of volicitin to wounded leaves led to an increase in emission of indole and sesquiterpene volatiles (Figure 1). The predominant sesquiterpenes in BAW-infested and volicitin-treated maize (cv Delprim), include β-caryophyllene, (E)-α-bergamotene, and (E)-β-farnesene. Thus, an upregulation of genes involved in sesquiterpene synthesis was predicted. Although measurement of volatiles occurred 12 hr after mechanical damage and volicitin addition, plants were harvested after only 3 hr for RNA isolation, since we assumed that maximal mRNA production of sesquiterpene genes would precede the maximal production of volatiles. Total RNA was extracted (Chang et al., 1993) and a subtracted cDNA library was produced (see "Methods and Materials"). To confirm differential expression of the isolated cDNAs, individual clones were hybridized with ^{32}P labeled cDNA from forward-subtracted and reverse-subtracted probes. Forward-subtracted probe is enhanced for volicitin-induced cDNAs, while the

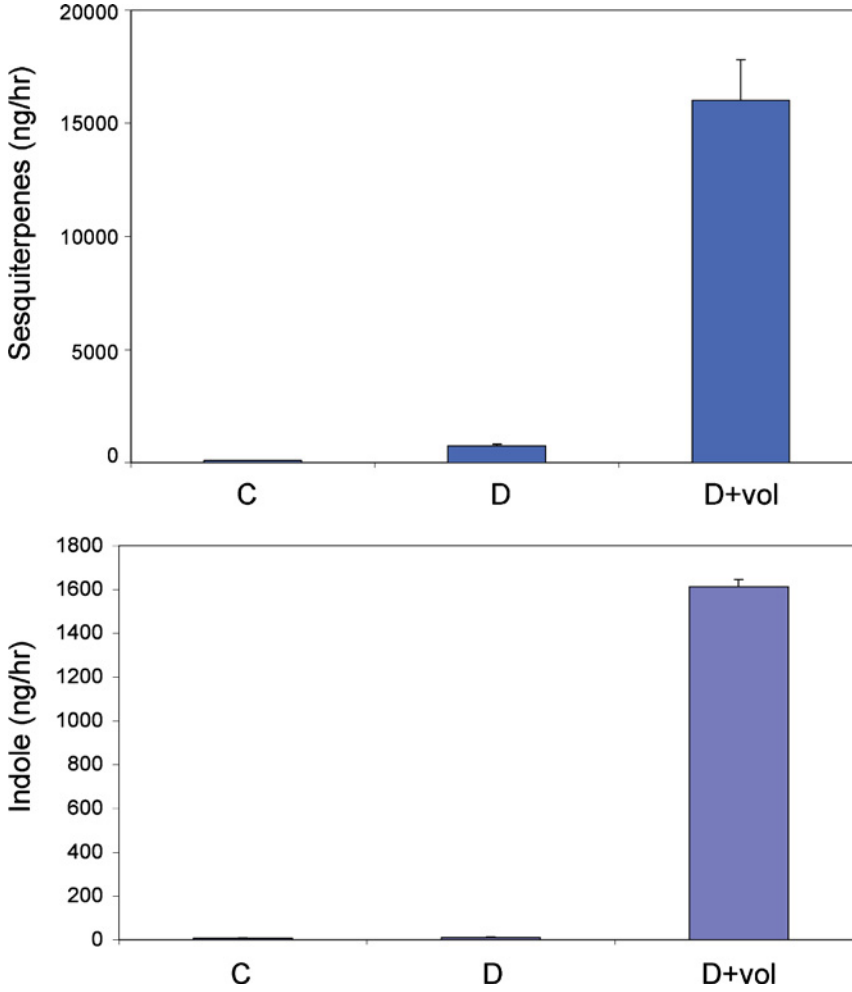

FIG. 1. Sesquiterpenes and indole are induced by volcitin treatment. Five nanomoles of volicitin was added to mechanically damaged leaves. C, Control; D, damaged; D+Vol, damage plus 5 nmol volicitin.

reverse-subtracted probe is enhanced for wound-induced cDNAs. Measurement of the radioactivity hybridizing to the individual clones using a phosphorimager allowed calculation of the ratio of the forward-subtracted cDNA divided by the reverse-subtracted cDNA. Values over 1 suggest that the clone is more abundant in the volicitin-treated sample, while values under 1 imply that the clone is more abundant in the wounded sample.

TABLE 1. GENES FOUND TO BE DIFFERENTIALLY EXPRESSED BY VOLICITIN TREATMENT

Ratio	Gene	Homology (tblastX)	Accession #	*E* value
>2	7	*Oryza sativa* putative sesquiterpene cyclase 1 (blastX)	BAB63870	8.00E–26
>2	8	No homology		
>2	9	*Oryza sativa* GDP dissociation inhibitor protein OsGDI1	AF016896	7.00E–59
>2	14	*Z. mays* CL39675_1 mRNA sequence	AY110070	3.00E–17
>2	15	*Z. mays* PCO129667 mRNA sequence	AY106046	1.00E–142
3.4	16	Salicylic acid carboxyl methyltransferase	AB049752	9.00E–75
9.2	18	*Z. mays* sesquiterpene cyclase 1 mRNA, Stc1	AF296122	2.00E–50
3.7	22	*Lycopersicon esculentum* GcpE mRNA	AF435086	0
3.4	24	*Lycopersicon esculentum* Pto kinase interactor 1	U28007	1.00E–163
0.3	25	*Arabidopsis thaliana* metalloprotease -related	NM_112804	1.00E–118
3.6	26	*Oryza sativa* clone OJ991214_12	AL606453	4.00E–44
3.3	28	Oryza sativa methylthioadenosine/S-adenosyl homocysteine nucleosidase	AF458088	4.00E–82
>2	29	*Oryza sativa* putative sesquiterpene cyclase 1	NM_183909	3.00E–42

Note. The ratio refers to the amount of volicitin-induced cDNA that hybridizes divided by the amount of wound induced cDNA that hybridizes to the clones. Unless otherwise noted, the tBlastX program was used to determine homologies and *E* values. Accession numbers are for the genes homologous to the maize genes.

Differential Gene Expression. Sixty-three differentially expressed clones were sequenced and organized into 27 genes (Table 1). Six of the genes had a ratio below 1 and 5 of these are associated with the chloroplast genome, suggesting that plastid-associated genes might be inhibited by volicitin treatment. However, when the chloroplast-associated genes were used as probes on virtual northern blots, they hybridized to numerous bands, and due to the complexity of the banding patterns it was not possible to confirm whether they are differentially expressed (data not shown). The complexity of the banding patterns was probably due to the considerable processing events typical of these polycistronic chloroplast messages (Buchanan et al., 2000). The cDNA was initially produced from total RNA using a primer with $T_{(30)}$ near the 3′ end, so it was initially surprising to find clones of chloroplast mRNAs since poly A+mRNA is not usually associated with chloroplast transcripts. However, messages containing short oligo A+runs have been noted in chloroplast genes (Grierson and Covey, 1984). In addition, chloroplast messages can have poly(A)-rich sequences added post-transcriptionally, which

make them particularly unstable and destined for degradation (Lisitsky et al., 1996). These two explanations may help to explain why six genes have sequences associated with the chloroplast genome. Considering the large amount of chloroplasts in leaf tissue, chloroplast RNA should represent a large population of the mRNA. Since we could not confirm using northerns whether these genes were differentially expressed, they were not included in Table 1.

Virtual northerns, which use cDNA rather than RNA, were used to confirm differential expression and to test whether BAW feeding induces the expression of these clones (Figure 2). Clones from 11 genes were examined on virtual northerns. Gene13 was below the detection limit of the virtual northern blot. Wounding induced the expression of nine of the genes (9, 18, 28, 15, 16, 26, 7, 29, and 24 see Figure 2), and volicitin enhanced the expression of RNA compared to the wounded sample in seven of them (18, 22, 16, 26, 7, 29, and 24). Leaves were subjected to BAW feeding for 6 or 18 hr; the expression of transcripts for these genes was induced in only four cases (18, 25, 7, 29). In five of the clones, less RNA was present in the BAW-infested sample compared to the control (9, 28, 15, 26, and 24). Consequently, four genes were induced by both volicitin and BAW feeding (18, 16, 7, and 29), while two genes were induced by volicitin but inhibited by BAW infestation (26 and 24). Finally, four genes were induced by wounding but inhibited by BAW feeding (9, 15, 26, and 24). Finding two genes that were induced by volicitin treatment but inhibited by BAW infestation was unexpected. Perhaps there is an additional component of BAW saliva that causes this inhibition of these genes. Indeed, Halitschke et al. (2003) found that four clones that were down regulated by regurgitant were induced by a FAC isolated from that regurgitant. In conclusion, although seven of the genes were induced by volicitin treatment, RNA was also found in the wounded sample. Consequently, none of the genes were specifically induced by volicitin.

Two genes may encode defense-signaling proteins. A rab-specific GDP-dissociation inhibitor (rab-GDI) from rice is similar to gene 9. The rice protein, found in fungal-elicitor- or salicylic acid-induced suspension cells, is involved in membrane trafficking, and is thought to be associated with defense signal transduction (Kim et al., 1999). This particular gene was only wound induced and was inhibited by BAW feeding (Figure 2), suggesting that this pathogen-related gene is turned off by insect infestation. Gene 24 encodes a protein similar to tomato Pto kinase interactor 1. This is a serine/threonine kinase that is phosphorylated by Pto. Both of these genes are part of the signaling cascade leading to the hypersensitive reaction in tomato caused by an interaction with bacterial speck (Zhou et al., 1995). Although this gene was induced by volicitin, it was also not on during BAW feeding (Figure 2). Another component of the BAW saliva may help to differentiate this pathogen-associated gene.

Gene 28 is similar to a putative methythioadenosine/*S*-adenosyl homocysteine nucleosidase from rice. This particular enzyme has been characterized in

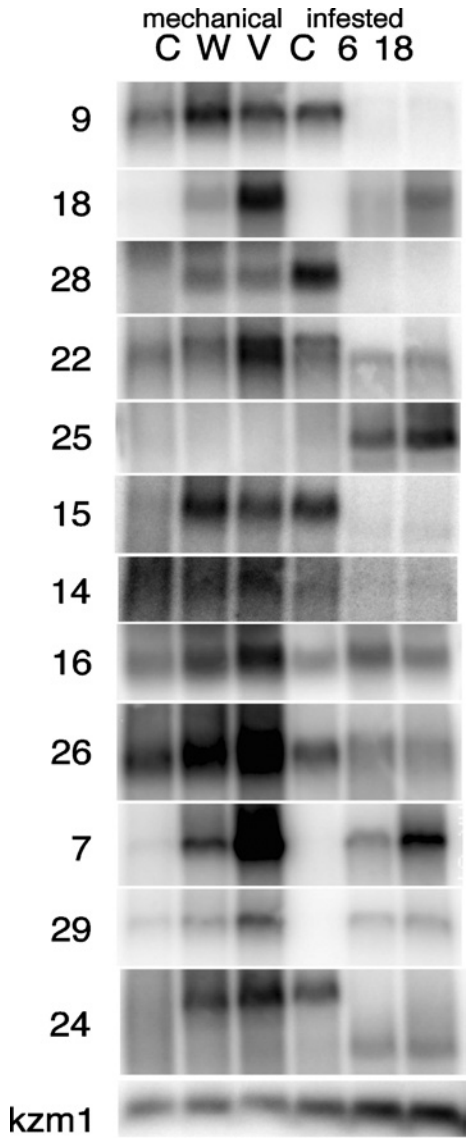

FIG. 2. Volicitin treatment and BAW feeding affected the levels of transcript present in maize leaves. cDNA from the following plant material was probed with various genes identified in Table 1. C, Control; W, wounded; V, wounded plus 5 nmol volicitin was added to leaves that were harvested after 3 hr. Maize leaves were (C) uninfested for 6 hr, or infested with BAW for 6 or 18 hr. *kzm1* constitutively expressed potassium channel gene from maize.

bacteria and purified from plants, where it hydrolyzes the glycosidic bond of 5'-deoxy-5'methyladenosine and *S*-adenosylhomocysteine to produce adenine and methylthioribose or *S*-ribosyl-homocysteine (Cornell et al., 1996). In bacteria, it is believed to have a role in salvaging methionine and adenine (Cornell and Riscoe, 1998). This particular gene was wound induced, while it was inhibited by BAW feeding (Figure 2). It is difficult to speculate about what this may mean considering that little is known about the function of this gene in plants.

Five genes are most likely related to the synthesis of volatile compounds. Monoterpenes (C10) and diterpenes (C20) are produced in the plastid, while sesquiterpenes (C15) are created in the cytosol. Two separate pathways are responsible for these compounds, which use the precursors dimethylallyl diphosphate (DMADP) and isopentyl diphosphate (IPP) (Aubourg et al., 2002). Three different genes (7, 18, and 29) have similarity to sesquiterpene cyclase genes responsible for the synthesis of sesquiterpenes. The three genes are most similar to three different genes in the database (Table 1), suggesting that they encode enzymes producing different compounds. An alignment of the sesquiterpene cyclase genes is shown in Figure 3. The three genes are compared with putative genes from *Arabidopsis*, and the two genes that have been characterized in maize (*stc1* and *tps1*). The *tps1* gene encodes an enzyme that produces $(E) - \beta$-Farnesene, a major component of the volatiles found in volicitin-induced Delprim maize. A 250 amino acid section of the coding region was compared with these genes. A phylogenetic tree of the sesquiterpene cyclases is shown in Figure 4. There was 47.9% identity between genes 7 and 29. There was only 14% and 12.8% identity between genes 18 and 7 or 29, respectively. Genes 7, 18, and 29 had 9%, 22.4%, and 8.6% identity with *tps1*, while they had 12.8%, 49.0%, and 14.0% identity with *stc1*. Clearly, gene 18 is most similar to *stc1*, while genes 7 and 29 are most similar to each other. This similarity is examined further with the phylogenetic tree in Figure 4. Bootstrap analysis is shown at the nodes, and is a measurement of the percent reliability that the branches are associated. For example, genes 29 and 7 are evolving from a common ancestor of *tps1*. This result was unexpected. However, the tree compared similar amino acids rather than just the percent that were identical. Consequently, the tree shows the similarity in sequences of genes 7 and 29 with *tps1*. Perhaps this suggests that there is a great deal of evolutionary pressure on these sequences to remain similar because they produce similar compounds. These genes may be responsible for the increase in CS that is seen with the addition of volicitin.

Gene 22 had an *E* value of zero with a GcpE protein from tomato, meaning that the protein sequence encoded by the gene is nearly identical to the sequence in tomato. The GcpE protein is involved in the synthesis of *DMADP* and *IPP* in the plastid (Querol et al., 2002). This suggests that the ultimate product of this reaction would be monoterpenes or diterpenes. The oxygenated monoterpene linalool is a predominant component of the volatile mixture. While the RNA from this gene was induced by volicitin, it was unaffected by BAW

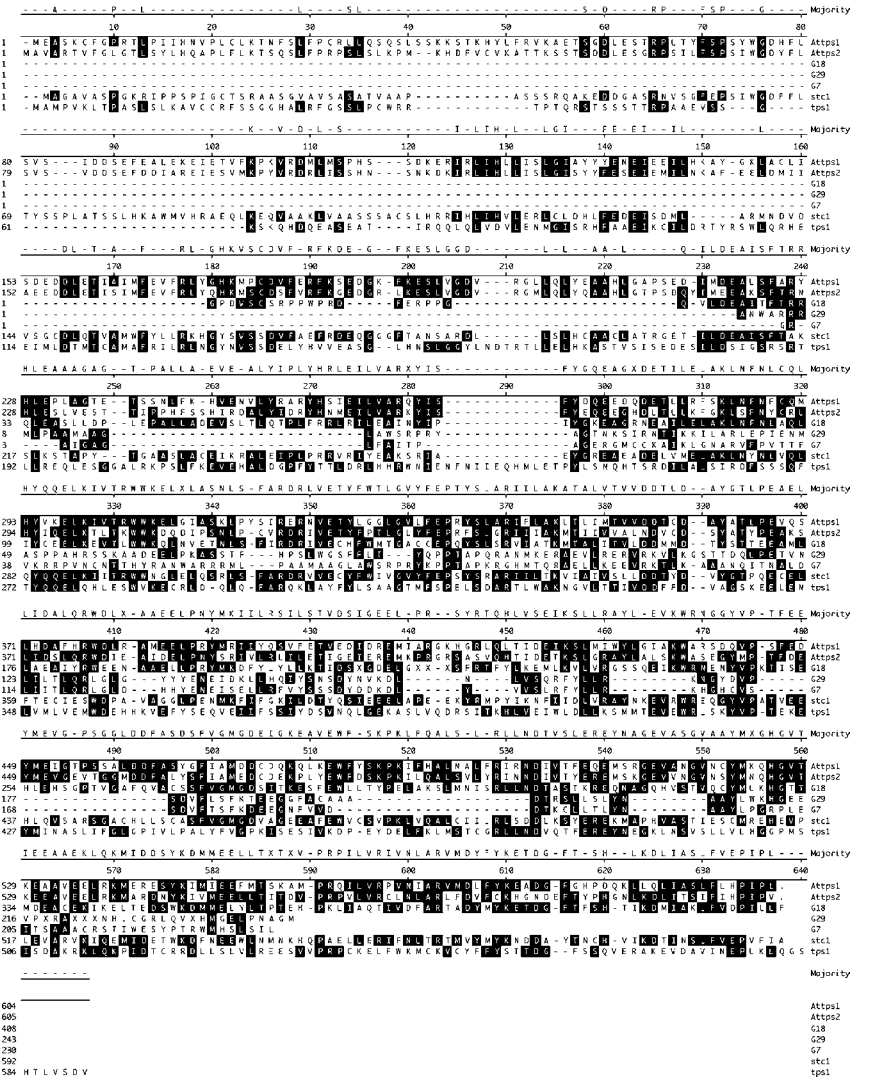

FIG. 3. Alignment of genes 7, 18, and 29 from Table 1 with other sesquiterpene cyclase genes shows similarity of these genes. *Attps1*, *Arabidopsis* At3G29410; *Attps2*, *Arabidopsis* At3G32030; *stc1* and *tsp1*, *Z. mays* sesquiterpene cyclase and terpene synthase.

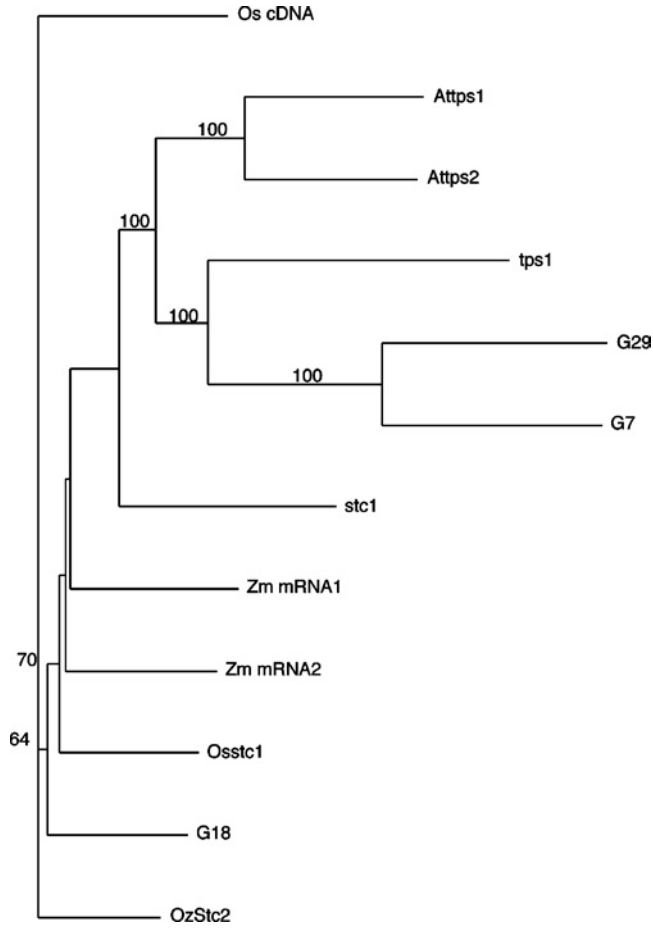

FIG. 4. Phylogenetic tree of most similar sequences with genes 7, 18, and 29 shows evolutionary relationship of these genes. Neighbor joining and distances program of PAUP was used for phylogenetic analysis. Bootstrap analysis (a measure of the percent reliability that the branches are associated) is shown at the nodes. OscDNA, *Oryza sativa* AK108761; *Attps1*, *Arabidopsis thaliana* At3G29410; *Attps2*, *Arabidopsis thaliana* At3G32030; *tps1*, *Z. mays* AAO18435; *stc1*, *Z. mays* AAK73113; maize mRNA1, *Z. mays* PC0101634; maize mRNA2, *Z. mays* PC0100777; Osstc1, *Oryza sativa* BAB63870; Osstc2, *Oryza sativa* BAC99549.1

feeding (Figure 2); additional components of BAW saliva may be responsible for this result. Gene 16 was also involved with volatile production. It is similar to *S*-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase (SAMT). This enzyme is responsible for the conversion of salicylic acid to methyl salicylate, a volatile compound that is responsible for chemoattraction of moths during pollination (Zubieta et al., 2003). However, a role in defense signaling cannot be ruled out since this substance plays a role in inter- and intra plant communication upon pathogen damage. This transcript of this gene was induced by both volicitin and slightly induced by BAW infestation (Figure 2). Considering that other pathogen-associated genes are inhibited by BAW feeding, this suggests that this gene may be involved in chemoattraction.

Volicitin-induced genes identified here are not specifically induced by volicitin or BAW feeding since they are also present in wounded tissue. The only transcripts that were found to be induced by both volicitin and BAW feeding were genes associated with volatile production. Perhaps, this is an indication of the specificity of the genes in the interaction with BAW. This suggests that a complex promoter might be present in these genes, since they are induced by both wounding and volicitin. Our finding of genes that are induced by volicitin but inhibited by BAW infestation was surprising, and suggests an added complexity to the promoters of these genes. An additional factor found in BAW/maize interaction is suggested by this result. The inhibition or lack of response of genes by BAW that are in turn induced by volicitin points to the idea that the plant is evolving in response to a complex mixture in the regurgitant. Consequently, for some genes, an induction by volicitin does not mimic the plant's interaction with BAW. In the plant's response to insect attack, the inhibition of some genes supercedes the effect of volicitin. Given that the induction of maize genes involved in volatile production is an advantage to the plant, it is unclear what volicitin confers for BAW. However, induction of plant defense genes due to a specific component of the pathogen is observed in plant/pathogen interactions.

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DIET-MEDIATED INTER-COLONIAL AGGRESSION IN THE FORMOSAN SUBTERRANEAN TERMITE *Coptotermes formosanus*

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Abstract—In most social insects, intercolonial and interspecific aggression are expressions of territoriality. In termites, cuticular hydrocarbons (CHCs) have been extensively studied for their role in nestmate recognition and aggressive discrimination of nonnest-mates. More recently, molecular genetic techniques have made it possible to determine relatedness between colonies and to investigate the influence of genetics on aggression. In the Formosan subterranean termite, *Coptotermes formosanus*, however, the role of CHCs and genetic relatedness in inter-colony aggression has been ambiguous, suggesting the involvement of additional factors in nest-mate recognition. In this study we assess the range of aggression in this termite species and characterize the influence of genetic relatedness, CHC profiles and diet on aggression levels. We collected four colonies of *C. formosanus,* feeding either on bald cypress or birch, from three locations in Louisiana. Inter-colony aggression ranged from low to high. Differences in CHC profiles, as well as genetic distances between colonies determined by using microsatellite DNA markers, showed no significant correlation with aggression. However, termite diet (host tree) played a significant role in determining the level of aggression. Thus, two distantly related colonies, each feeding on different diets, showed high aggression that significantly diminished if they were fed on the same wood in the laboratory (spruce). Using headspace solid phase microextraction, we found three compounds from workers fed on birch that were absent in workers fed on spruce. Such diet-derived chemicals may be involved in the complex determination of nest-mate recognition in *C. formosanus*.

Key Words—Cuticular hydrocarbons, microsatellite DNA markers, agonism, termite.

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INTRODUCTION

The Formosan subterranean termite, *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae), was introduced into the continental United States in wooden cargo crates and pallets shipped from the Asian theater following World War II (La Fage, 1985). Since it was initially discovered, *C. formosanus* has spread to several southern states and causes over $1 billion per year in damage and control costs (Su and Scheffrahn, 1990). Investigations of this pest have primarily focused on methods of control such as chemical and baiting technologies. Although some studies have focused on biological issues, including aggression and colony interactions, results have been ambiguous.

Many social insects show territoriality in defending their resources from other insects (Wilson, 1971). Among termite species, agonistic behaviors include fighting, fleeing, or submission, and such behaviors occur between individuals from different colonies of the same or different species (Haverty and Thorne, 1989; Thorne and Haverty, 1991). Factors responsible for agonistic behavior in termites are not fully understood, especially in *C. formosanus*. Several factors that could be involved include cuticular hydrocarbons (CHCs), glandular secretions, or pheromones, as well as environmental factors (Su and Haverty, 1991).

It has generally been held that CHCs of insects play a role in interspecific and intraspecific recognition (Blomquist et al., 1987). There is a growing body of evidence suggesting that termites have species-specific mixtures of CHCs (Page et al., 2002). Also, a correlation between aggressive behavior and hydrocarbon phenotypes was reported among *Zootermopsis* spp. (Haverty and Thorne, 1989), and CHC phenotypes and their relationship to agonism have been extensively studied among species of *Reticulitermes* (Haverty et al., 1999; Delphia et al., 2003). Additionally, genetic components have been implicated in inter-colonial recognition, as is the case in *Microcerotermes arboreus* (Adams, 1991) and *Schedorhinotermes lamanianus* (Husseneder et al., 1998).

Like other termite species, *C. formosanus* also exhibits inter-colony agonism, but the behavior is not always consistent. In a study of *C. formosanus* from cypress trees in Lake Charles, LA, no agonism was observed between different colonies (Delaplane, 1991). The author suggested that high genetic relatedness may have caused the lack of agonism. Aggression between colonies of Hawaiian *C. formosanus* was apparently not correlated with inter-colonial relatedness, i.e., CHC data did not correlate with agonistic behavior (Su and Haverty, 1991). CHCs have been characterized from numerous colonies of *C. formosanus*, and statistically significant quantitative differences have been found between workers and soldiers, between colonies, and for different sampling months (Haverty et al., 1996). A series of alternative mechanisms were listed for nest-mate recognition in *C. formosanus* (Su and Haverty, 1991), collectively called the "multiple stimulus hypothesis," which essentially states that recognition may depend on a complex

set of behaviors and stimuli that act together in determining nest-mate recognition (Thorne and Haverty, 1991).

In the present study, after identifying colonies of *C. formosanus* that exhibited either strong agonism or no agonism, we investigated factors that might be responsible for the induction of the observable agonistic behavior. Such factors included cuticular hydrocarbon profiles, genetic relatedness of colonies, and diet.

METHODS AND MATERIALS

Termites. Four colonies of *C. formosanus* were collected to determine the range of inter-colonial aggression. Two of these, located approximately 430 meters apart, were collected from bald cypress, *Taxodium distichum*, trees in City Park located in New Orleans, LA, and designated CP-2 and CP-3. The third colony was collected from a river birch, *Betula nigra*, tree on the University of New Orleans campus and named UNO. A fourth colony was collected from a bald cypress tree in Sam Houston State Park in Lake Charles, LA (approximately 320 km west of New Orleans) and named LC. With the exception of the LC colony (which was maintained on blocks of pine, *Pinus* sp., wood in the laboratory for approximately, 6 mo before use), and those termites subsequently used in determining the effect of diet (maintained on either spruce, *Picea* sp., or birch, *Betula* sp., wood for three months), termites were collected directly from their host trees immediately prior to use in aggression assays, chemical analysis, or genetic tests.

Aggression Assays. For short-term (5 min) assays, a Whatman No. 4 filter paper (90 mm diam) placed in a 100 × 15 mm Petri dish (Becton Dickinson, Franklin Lakes, NJ) was sprayed with a fluorescent solution [1.5 g Blue Luminous Powder (BioQuip, Gardena, CA) suspended in 500 ml distilled water] and dried in an oven at 60°C. The filter paper was moistened with 1 ml distilled water and ten workers of at least third instar and one soldier from a test colony were allowed to walk in the dish for 1 min to ensure sufficient acquisition of the powder on their appendages and abdomen. The marked termites were then released into a 50 × 9 mm Petri dish (Becton Dickinson) with a 42.5 mm diam Whatman No. 4 filter paper moistened with 200 μl distilled water. Ten unmarked workers and one soldier from either a different colony or the same colony (control) were then released into the same Petri dish.

The dish was illuminated using a 45 cm fluorescent blacklight and placed under a color CCD camera (Emcal Scientific, Poway, CA) to distinguish between marked and unmarked termites. Behavior was recorded for 5 min. using a Panasonic AG-6740 time-lapse video recorder. All possible colony pairings and controls were replicated ten times. The colonies were alternately marked and unmarked resulting in five marked and unmarked trials for each colony. In a second experiment, to determine the effect of marking on aggression, 10 replicates

consisting of ten unmarked workers and one soldier paired with 10 marked workers and one soldier from the same colony served as control pairings.

The number of aggressive behaviors were observed and recorded for each pairing. Aggressive encounters included only those in which attempts at inflicting damage upon another individual were noted. That is, mandibles were open and a lunging action was made toward another individual of the opposite colony, or workers chased individuals from a different colony in an attempt to inflict bites. Behavior was not considered aggressive if mandibles were simply open without aggressive maneuvers. The mean and standard error of the number of aggressive behaviors occurring within 5 min were calculated for each set of pairings. For 24 hr assays, (long-term aggression assays), colony pairings used in short-term tests were removed from beneath the CCD camera and placed in an incubator maintained at 28 $\pm$ 1°C, 80% RH and constant darkness. After 24 hr, the total number of dead individuals was recorded and converted to percentage mortality. Average and standard error for the percentage mortality were calculated for each colony pairing.

Genetic Analyses. Workers from each test colony were preserved in 95% ethanol and shipped to the University of Hawaii, Honolulu, HI for DNA extraction. Individual DNA was extracted and purified from up to 20 workers per colony using the DNeasy Kit (Qiagen, Inc., Chatsworth, CA). Purified DNA was then shipped to Dr Edward Vargo, North Carolina State University, for genetic analyses using microsatellite DNA markers (Vargo and Henderson, 2000; Husseneder et al., 2003; Vargo, 2003). To describe the social organization and differentiation of the colonies, eight microsatellite loci were scored using a subset of markers developed by Vargo and Henderson (2000): Cf 10-4, Cf 10-5, Cf 12-4, Cf 8-4, Cf 4:1A2-4, Cf 4-10, Cf 4-9A, Cf 4-4. A group of individuals (16.8 $\pm$ 4.3, mean $\forall$ SE) were genotyped per locus and colony according to published protocols (Vargo and Henderson, 2000).

Cuticular Hydrocarbon Profiling. Through preliminary GC-MS analysis, 0.232 g (approximately 50 CP-3 workers) was determined to be an ideal weight for extraction. Consequently, 0.232 g of workers (37–72 workers depending on colony) from each colony were placed in 5 ml glass vials capped with Teflon inserts. Workers were extracted in 2.0 ml of hexane (J. T. Baker, Phillipsburg, NJ) for 10 min, and the extract was filtered through a 0.45 μm PTFE filter using a 1.0 ml glass syringe. The eluate was collected in a 2 ml vial, evaporated under nitrogen to 100 μl, and transferred to a glass insert contained in an autosampler vial. Analyses for hydrocarbons were carried out as reported by Bland et al. (2001). Briefly, 2 μl of each extract were analyzed by GC-MS using an Hewlett-Packard 6890 Series GC System with a 5973 Mass Selective detector and connected to an Hewlett-Packard Kayak XA computer interface. The inlet temperature was set at 250°C, and samples were injected in splitless mode. Chromatograms were run with a constant flow of 1 ml/min of ultrapure helium gas. A DB-5 column

(30 m × 0.25 mm, 0.25 μm film thickness, J&W Scientific, Folsom, CA) was used with temperature programming from 60°C with a 1 min hold, to 300°C at 10°C/min with a final 10 min hold. Previously determined hydrocarbon profiles (Bland et al., 2001) were used to identify CHCs in all chromatograms.

Effect of Diet on Aggression. UNO and CP-3 termites were collected from their respective colonies. Workers and soldiers from UNO were placed on birch (UNO-birch) or spruce (UNO-spruce) wood blocks, and workers and soldiers from CP-3 were placed on spruce (CP-3-spruce) wood blocks for three months. Three sets of assays were performed. In one group, UNO-birch was compared with UNO-spruce. The second group included UNO-birch vs. CP-3-spruce, and the third group included UNO-spruce vs. CP-3-spruce. For each assay, ten workers and one soldier, marked or unmarked, from each colony were tested for mortality over a period of 24 hr as described above. Each pairing was replicated five times, and mortality was recorded after 24 hr.

Solid Phase Microextraction (SPME) Analysis. Because previous hexane extractions showed no significant qualitative differences in CHC composition among colonies, a different method of chemical analysis, solid phase microextraction, was performed (Bland et al., 2001). Workers (0.037 g) from each sample of UNO-birch, UNO-spruce, and CP-3 spruce were placed into separate vials with a septum. Five types of SPME fibers [100 μm polydimethylsiloxane (PDMS), 65 μm polydimethylsiloxane-divinylbenzene (PDMS-DVB), 70 μm Carbowax, 50/30 DVB/Carboxen/ PDMS, 75 μm Carboxen-PDMS] used in this experiment were obtained from Supelco (St Louis, MO). Each SPME fiber was inserted into the vial at a point 1 cm above the termites. The vial, with the SPME fiber, was placed in a sand filled heating block maintained at 120°C for 60 min. SPME samples were analyzed by GC-MS as described above. Mass spectra were recorded from 40 to 750 m/z. Compounds of interest were identified by mass spectral matches with Wiley database entries and retention time and by mass spectral comparison with authentic samples obtained from Sigma–Aldrich (St Louis, MO).

Statistical Analyses. General descriptive statistics of the colony genetic structure, such as numbers of alleles per locus, number of private alleles, and allele frequencies were calculated for each colony and locus using the program GDA (Lewis and Zaykin, 2000). To assess genetic differentiation between colonies, we first searched for private alleles, i.e., alleles that are exclusively found in one of the colonies and not in others. Second, we used Fisher's exact test of genotype frequency differentiation among colonies using GENEPOP (Raymond and Rousset, 1995). Third, genetic distances (F_{ST}) were calculated between colonies using the methods of Weir and Cockerham (1984) as implemented in FSTAT (Goudet, 1995). Colonies were treated as subpopulations (Thorne et al., 1999; Bulmer et al., 2001). To assess the significance of the F-statistics, 95% confidence intervals (CI) were constructed by bootstrapping over loci. F-values were

considered significantly different from zero if their confidence intervals did not span zero.

To determine statistically significant differences in aggression and mortality among pairings of colonies, a one-way analysis of variance (ANOVA) and a Tukey's honestly significant difference (HSD) test were performed on the data using SPSS 11.0 statistical analysis software (SPSS Inc., Chicago, IL) Tests were performed using a significance level of $\alpha = 0.05$. Pairwise F_{ST} values were used as genetic distances for analysis of correlation between short-term aggression levels and mortality after 24 hr using a Mantel test (Mantel, 1967).

RESULTS

Aggression Assays. Short term aggression assays (Figure 1A) showed aggression resulting in injury between individuals from all colonies collected from the field except between pairings of CP-2 and CP-3. No aggressive behaviors were observed in any of the pairings from the same colony (control). The highest numbers of short term aggressive behaviors occurred in UNO vs. CP-3 (50 ± 3.8) and UNO vs. LC (42 ± 3.0). Moderate aggression was observed in UNO vs. CP-2 (28 ± 2.3), CP-3 vs. LC (20.1 ± 2.5), and CP-2 vs. LC (13.1 ± 1.3). There was essentially no aggression observed in CP-3 vs. CP-2 (0.20 ± 0.13). ANOVA indicated that, overall there was a significant effect of colony pairing on aggression levels ($P < 0.001$). *Post hoc* tests using Tukey's HSD test showed that the only pairings not significantly different in levels of short term aggression were UNO vs. CP-3 and UNO vs. LC ($P = 0.212$); UNO vs. CP-2 and CP-3 vs. LC ($P = 0.223$), and CP-3 vs. LC and CP-2 vs. LC ($P = 0.348$). However, all pairings were significantly different from the control pairings, except for the pairing of CP-3 vs. CP-2 ($P = 0.166$).

Although both CP-2 vs. LC and CP-3 vs. LC showed only moderate short term aggressive behaviors, these pairings had the greatest mortality after 24 hr (Figure 1B). UNO vs. CP-3, which had the greatest number of short term aggressive encounters, also had the third greatest percentage mortality. Although, UNO vs. CP-2 showed an average of 14 fewer aggressive encounters than UNO vs. LC in short term assays, both pairings had approximately the same average percent mortality after 24 hr (57.8% and 60.5%, respectively).

Overall, there was a significant effect of colony pairing on long-term aggression ($P < 0.001$). However, some individual group pairings including UNO vs. CP-2 and UNO vs. LC ($P = 0.999$); CP-3 vs. LC and CP-2 vs. LC ($P = 0.999$); CP-2 vs. LC and UNO vs. CP-3 ($P = 0.999$), and CP-3 vs. LC and UNO vs. CP-3 ($P = 0.991$) were not significantly different in levels of mortality. All colony pairings had significant differences in levels of mortality vs. controls except for CP-3 vs. CP-2, which had P-values of 0.112 vs. UNO and CP-2 controls and 0.053 for CP-3 and LC controls.

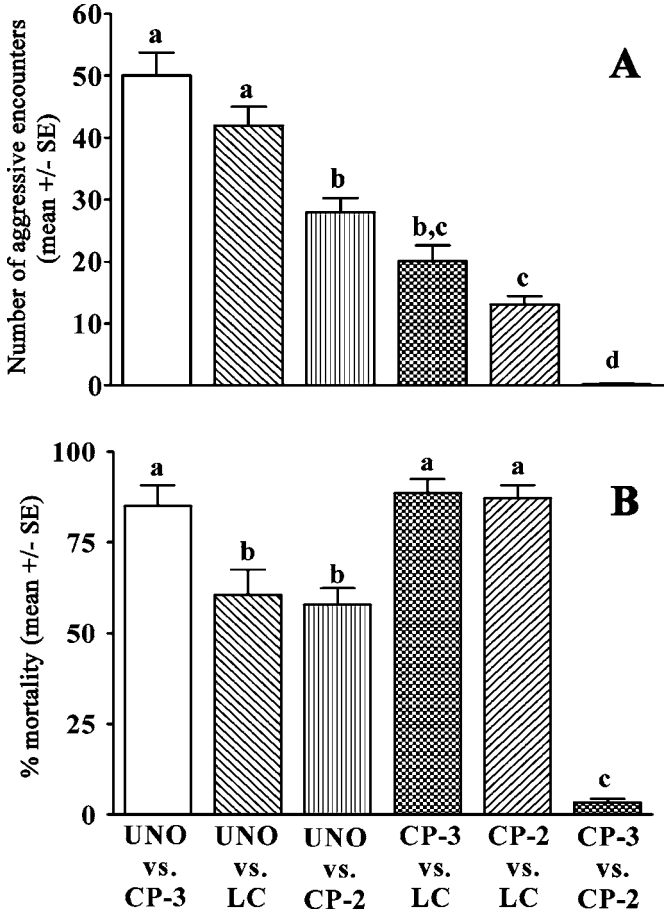

FIG. 1. Results from aggression assays among individuals of four test colonies of *C. formosanus*. (A) Short-term aggressive encounters over 5 min. (B). Mortality after 24 hr. Mean ± SE. Means with the same letters are not significantly different from each other at $\alpha = 0.05$ ($N = 10$).

Genetic Analyses. All eight microsatellite loci were polymorphic with three to five alleles per locus and allele frequencies ranging from 0.01 to 0.54 across all colonies. Microsatellite analysis showed that the four colonies were genetically distinct. First, private alleles were found in each of the colonies, distinguishing UNO, LC (both with private alleles at three different loci), CP-2, and CP-3 (both with different private alleles at the same locus). Second, Fisher's exact test confirmed that the four colonies were significantly differentiated at all loci ($P < 0.001$, $\chi^2 = \infty$, d.f. = 16). Third, F_{ST}, a measure of genetic contrast

between the colonies, was significantly different from zero ($F_{ST} = 0.38$, 95% CI = 0.30–0.46, nonoverlapping with zero). Pairwise F_{ST}-values ranged from 0.24 to 0.48. Neither short-term levels of aggression ($r = 0.2314$, $P = 0.659$) nor long-term levels of aggression (i.e., mortality) ($r = 0.449$, $P = 0.371$) were significantly correlated with genetic distance.

Chemical Profiles from Worker Extracts. Analysis of hexane extracts of workers sampled from the four colonies showed no qualitative differences between colonies, but did show quantitative differences in the amounts of some CHCs (Figure 2). LC and CP-2 had greater amounts of several CHCs compared to UNO and CP-3, with the exception of peak #2 (9-,11-,13-methylpentacosane), of which UNO had the greatest amount. CP-2 and LC had greater amounts of all the straight chain *n*-alkanes observed and identified in the chromatogram. The straight chain *n*-alkanes include peaks #1 (pentacosane), #5 (hexacosane), and #8 (heptacosane). The greatest differences in *n*-alkane hydrocarbon quantities can be seen in peaks #12 (octacosane) and #14 (nonacosane). These chromatograms show that there were greater amounts of octacosane and nonacosane present in samples from CP-2 and LC than in the other two colonies. CP-2 and CP-3 showed observable differences in CHCs based upon peaks 1, 5, 8, 12, 14, and unidentified peak 18.

Effect of Diet on Aggression. When workers from previously aggressive colonies were fed the same diet for 3 months, aggression between the colonies was significantly reduced. From the results (Figure 3), it was concluded that diet had a significant influence on inter-colonial aggression in *C. formosanus*. There was no significant difference in aggression between UNO-spruce vs. CP-3-spruce and UNO-birch vs. UNO-spruce ($P = 0.567$). When UNO- birch was paired against CP-3-spruce, mortality was over 90%, which is similar to levels of aggression seen in previous aggression assays involving UNO and CP-3. In this assay, when both UNO and CP-3 colonies were fed spruce wood, the intense levels of aggression towards each other greatly diminished. However, if diet alone were enough to manipulate aggression then the UNO-birch, should have been aggressive towards UNO-spruce. This was not the case in the pairing of UNO-spruce vs. UNO-birch, suggesting that although diet appears to influence intercolonial aggression there must be other factors involved that affect intra-colonial aggression.

SPME Analysis. Five different fibers were used for SPME analysis of the groups of termites from CP-3 fed on spruce and groups of termites from UNO fed on spruce or birch. Results indicated that in all analyses, three compounds, eluting between 5 and 15 min, were found to be in either substantially greater amounts or completely unique in the UNO-birch samples (Figure 4). Based upon the retention times and mass spectra, identification was made of each of the three compounds as dimethyltrisulfide (1), 2-aminoacetophenone (2), and 8-heptadecene (3). Mass spectra of the three authentic compounds after SPME, matched the compounds

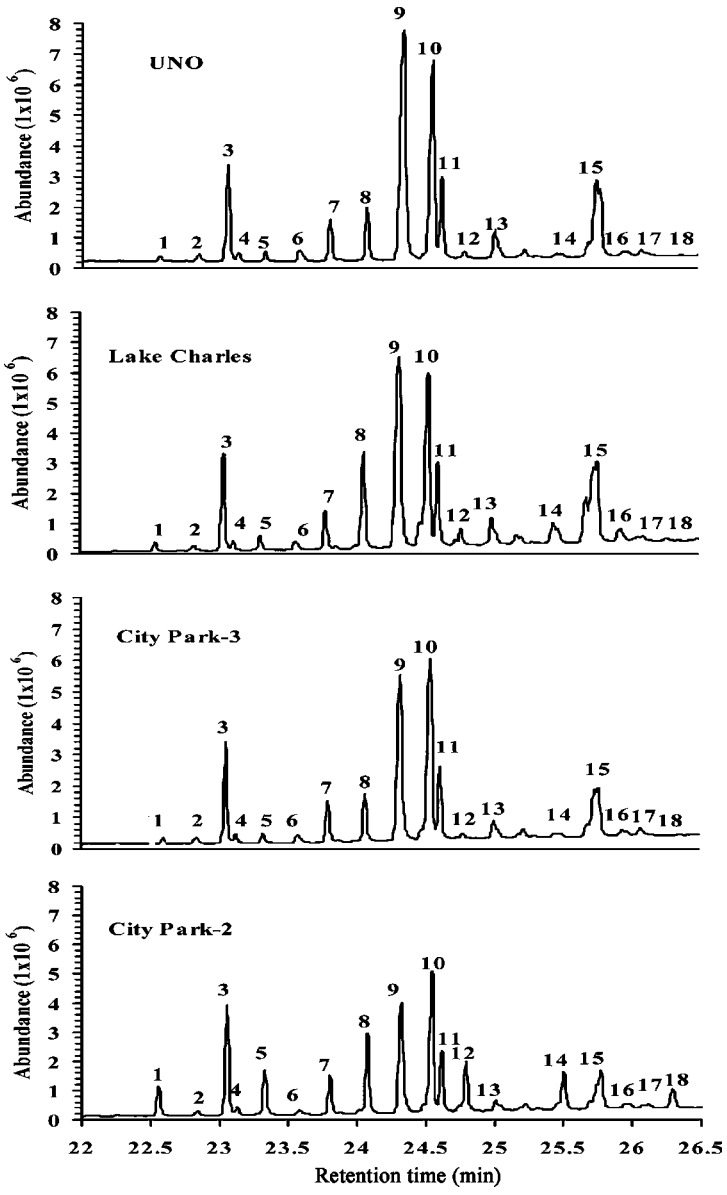

FIG. 2. Chromatograms of CHCs from workers of four test colonies of *C. formosanus*. No qualitative differences in CHCs were found. Quantitative differences in peaks 1,5,8,12,14, and 18 between CP-2 and CP-3 did not correlate with aggression.

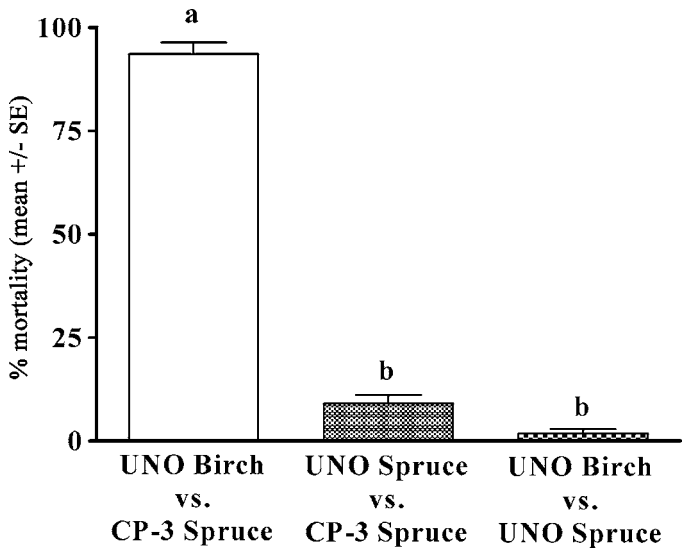

FIG. 3. Mortality after 24 hr as a result of aggression between two colonies of *C. formosanus* fed on spruce or birch woods. Mean ± SE. Bars with the same letters are not significantly different from each other at $\alpha = 0.05 (N = 5)$.

identified from the termites. Retention times and mass spectra were essentially the same for each compound across all five types of fibers used. No qualitative differences in hydrocarbon profiles were noted using different fiber types.

DISCUSSION

Intraspecific or intercolonial aggression has been studied in numerous genera of termites including *Hodotermes*, *Trinervitermes*, *Amitermes*, *Reticulitermes*, and *Zootermopsis* (Shelton and Grace, 1996 and references therein). Previous investigations involving *C. formosanus* showed that intercolonial pairings from Lake Charles, LA did not evoke significant aggression, and individuals could not accurately discriminate nest-mates from nonnest-mates (Delaplane, 1991). It was also reported that colonies of *C. formosanus* from Hallandale, FL showed no aggression towards individuals from other colonies from the same area. However, colonies located on the University of Hawaii campus were able to discriminate nest-mates from nonnest-mates in some pairings, and were variable in their levels of inter-colonial aggression (Su and Haverty, 1991). We also observed varying degrees of aggression in all paired combinations among the four test colonies, except for pairings between CP-2 and CP-3.

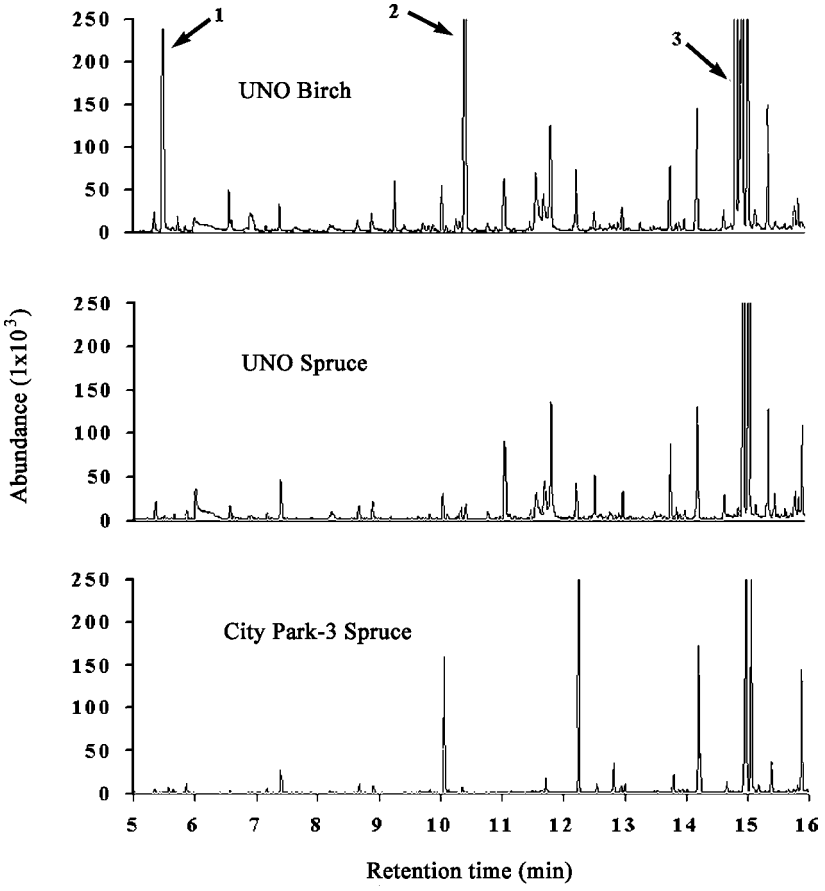

FIG. 4. Chromatograms of compounds obtained by headspace solid-phase microextraction of workers from two colonies of *C. formosanus* workers fed on spruce or birch wood. Three compounds [dimethyltrisulfide (peak 1), 2-aminoacetophenone (peak 2), and 8-heptadecene (peak 3)] associated with the UNO colony fed on birch were either absent or highly reduced in the other two colony/diet combinations.

Aggression could not be accurately described by short-term observation alone. For example, CP-2 and LC pairings resulted in only moderate short-term aggression. However, they displayed a high level of mortality with $87.1 \pm 3.6\%$ of individuals dead after 24 hr. Mortality after 24 hr ranged from 58% to 90% in five of the six pairings, whereas it was <4% in CP-2 vs. CP-3. Although no aggression was encountered between the two samples from City Park, microsatellite DNA analyses showed that all four colonies were genetically distinct.

Neither short-term levels of aggression nor mortality were significantly correlated with genetic distance. In the same way, using multilocus DNA fingerprinting, no correlation between genetic similarity and aggression was found in termites from 12 collection sites in Hawaii (Husseneder and Grace, 2001).Similarly, in an earlier study, differences in mitochondrial DNA of different colonies of *C. formosanus* could not be linked with agonism (Broughton and Grace, 1994). Thus, in contrast to other termite species (Adams, 1991), genetic traits cannot fully explain intercolonial patterns of aggression in *C. formosanus*.

Visual observation of the CHC profiles of each colony revealed that the CHC profiles did not appear to correlate with aggression. For example, although some quantitative differences were observed among CHC profiles of the workers from the four colonies if aggression in *C. formosanus* was correlated with quantitative differences in CHC profiles, then both CP-3 and UNO should act aggressively towards CP-2 and LC. However, CP-2 and CP-3, with clear quantitative differences in CHC profiles, were not aggressive to each other in behavioral assays. We did note some quantitative differences in CHCs between pairs of aggressive colonies. As example, the LC colony which showed high level of aggression towards all other colonies had higher amounts of straight chain *n*-alkanes (Figure 2, peaks 1, 5, and 8). Similarly, for UNO vs. CP-3, which exhibited high short-term aggression and mortality, the ratio between peaks 9 and 10 was reversed. Because CHC profiles in *C. formosanus* vary between castes within a colony (Haverty et al., 1996), it has been suggested that it is unlikely that CHCs would be used by this species as the sole cue of nest-mate recognition (Matsuura, 2001).

In contrast, experiments performed on numerous other species of social insects have shown that aggression is correlated with different CHC profiles. In the paper wasp, *Polistes dominulus*, it was reported that methyl-branched alkanes and alkenes were used as the chemical signature for nest-mate recognition (Dani et al., 2001). In dampwood termites, *Zootermopsis* spp., when individuals from different colonies containing different CHC phenotypes were paired, they typically showed high levels of aggression (Haverty and Thorne, 1989). On the other hand, pairings between termites of the same CHC phenotype showed consistently low levels of aggression or even no aggression. In *Reticulitermes* spp., pairing of individuals from different colonies with the same CHC phenotype resulted in little immediate aggression, but showed high mortality after 24 hr, whereas pairing of individuals from different CHC phenotypes resulted in immediate aggression in most pairings and approached 99% mortality after 24 hr (Haverty et al., 1999; Delphia et al., 2003). These authors concluded that *Reticulitermes* could differentiate CHC phenotypes and recognize nest-mates and nonnest-mates even within a CHC phenotype.

Our behavioral tests, which were designed to analyze the effect of diet on colony aggression, showed clear differences in the levels of aggression. When

UNO (originally from birch) and CP-3 (originally from cypress) were both fed on spruce, aggression was significantly reduced compared to the intense aggression observed previously between these two colonies. Few studies have focused on the influence of diet on aggression in social insects. Liang and Silverman (2000) determined that in the Argentine ant, *Linepithema humile* (Hymenoptera: Formicidae), hydrocarbons acquired from the feeding on insect prey are used in nest-mate recognition. Subsequently, Silverman and Liang (2001) determined that when colony fragments were separated for a period of time and provided prey that possessed distinct CHC profiles, these fragments were no longer able to reintegrate into the colony due to the high levels of aggression. These results indicated that diet could influence not only inter-colonial aggression, but also intra-colonial aggression. Although there was a limited number of pairings in our study, the results suggest that diet may influence inter-colonial aggression in *C. formosanus*, but not intra-colonial aggression. Similarly, when *S. lamanianus* workers were kept on nest materials from other colonies inter-colonial aggression diminished. However, it was not possible to induce intra-colonial aggression by placing workers on alien nest material (Husseneder et al., 1997). In listing a series of alternative mechanisms for nest-mate recognition in *C. formosanus*, collectively known as the "multiple stimulus hypothesis" (Haverty and Thorne, 1989), one of these mechanisms was suggested to be based on volatile digestive components (Thorne and Haverty, 1991). In *Reticulitermes speratus,* it has been suggested that differential intestinal bacterial composition (perhaps due to feeding on different foods) leads to production of colony-specific chemical cues responsible for nest-mate recognition (Matsuura, 2001).

The results of the SPME analyses indicate that a change in diet qualitatively altered the chemical signature of the termites in our study. Three compounds (dimethyltrisulfide, 2-aminoacetophenone, and 8-heptadecene) were found in groups of termites fed on birch but absent or in significantly lower amounts in groups fed on spruce. At this time, we are not sure if these chemicals were acquired from the host wood, were produced within the termite by the action of gut fauna, or were produced as a result of subjecting the termites to high temperature. When the termites were removed from birch and placed on spruce, the characteristic chemical signature changed. This may explain why individuals from CP-3 (cypress) and UNO (birch) placed together showed intense aggression, and when later they were reared on the same diet (spruce), aggression was greatly diminished. Thus, diet appears to be an important factor influencing intercolonial aggression in *C. formosanus*.

Although feeding on the same diet could reduce intercolonial aggression, feeding on different diets could not induce aggression within the same colony. Thus, when UNO-birch was paired with UNO-spruce, there was no aggression and no resulting mortality, even after 24 hr. Some genetic or other predisposition must exist which limits colony members from attacking one another, even when

they feed on different food sources. It appears that there must be an intricate process involving a hierarchy of cues that regulates nest-mate recognition in *C. formosanus*. This hierarchy may also explain why individuals from the UNO colony did not show intra-colonial aggression when reared on different diets. If they detect dissimilar diet-derived volatile components, they may then rely on a second level of recognition in which they verify endogenous genetically based colony-specific cues. This might represent a mechanism to maintain territoriality and, at the same time, lead to resource partitioning between different colonies of a termite species.

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ERRATUM

INTRASPECIFIC AND INTERSPECIFIC INTERACTIONS MEDIATED BY A PHYTOTOXIN, (—)-CATECHIN, SECRETED BY THE ROOTS OF *Centaurea maculosa* (SPOTTED KNAPWEED)

TIFFANY L. WEIR,* HARSH PAL BAIS, and JORGE M. VIVANCO

In the above paper, originally appearing in Vol. 29, No. 11, November 2003, Figure 1B should be replaced by the figure below. The initial figure showed a catechin concentration that was overestimated by approximately fivefold. In addition, the figure legend stated that the concentration units were mg/ml, which has been corrected to μg/ml. However, the overall trends reported in the data

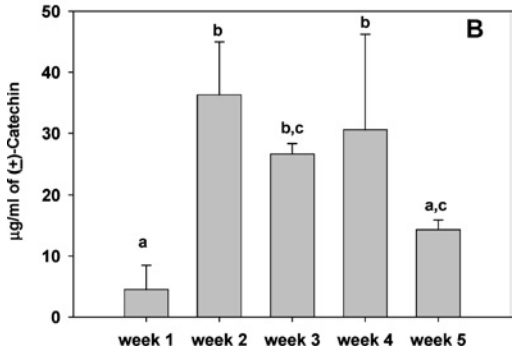

FIG. 1. (B) The amount of catechin (μg/ml) present in hexane-partitioned media extracts containing root exudates of *C. maculosa* were quantified by HPLC analysis. Error bars show +1 SD. Significantly different means are denoted by different letters; ANOVA, $P < 0.05$.

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have not been affected by this change. The authors believe that due to the large variation between repetitions and experimental limitations discussed in the original manuscript, these data do not accurately reflect average catechin secretion by individual plants; however, they do indicate that *C. maculosa* begins to secrete catechin very early in development and that this catechin secretion increases as the plant begins to mature.

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